SOME CONTRIBUTIONS TO THE STUDY OF THE MEGALOBLASTIC ANAEMIAS AND OTHER CONDITIONS ASSOCIATED WITH DISORDERED METABOLISM OF CYANOCOBALAMIN, PTEROYLGLUTAMIC ACID AND RELATED SUBSTANCES.

By

Ronald Haxton Girdwood, M.B., Ch.B.(Hons.), Ph.D.,

F.R.C.P.Ed., M.R.C.P.Lond.

FOREWORD

This thesis contains data collected during researches carried out in India while on Army Service under wartime conditions in 1944-45, in Britain since 1946, and in the United States during the period of tenure of a Rockefeller Fellowship in 1948-49.

The work described here is partly clinical, but much of it has been carried out in the laboratory. The investigations have had to be planned according to the material and facilities available at each centre and to be modified in the light of rapid advances in knowledge. At the time of the studies on Indian troops, for example, folic acid had not been synthesised and cyanocobalamin (vitamin B_{12}) was unknown. The laboratory then was a straw hut, the apparatus consisted chiefly of a microscope, a centrifuge and a Sahli haemoglobinometer, and the questioning had to be carried out largely in the Urdu language. The data collected then have been included here, however, as they draw attention to an unexpected problem of megaloblastic anaemia that gave rise to anxiety because of the extent to which it affected Indian troops fighting in the East in the Second World War. At the time our knowledge was insufficient to enable a biochemical study of the condition to be carried out and indeed much of this thesis concerns the development of satisfactory techniques for the study of these problems of 1942-45.

During the period of the investigations, pteroylglutamic acid,/

acid, cyanocobalamin, citrovorum factor, and the folic acid antagonists have all been produced and new methods for their study have been evolved. The investigation of the megaloblastic anaemias now necessitates consideration of biochemical problems of great complexity. Much of the investigative work recorded here has required the use of microbiological assay techniques which were soon found to be much less specific than had at first been believed. Accordingly the techniques have become more complicated, new methods of approach have had to be devised, and interpretations of the results have had to be made with caution.

The prevention of megaloblastic anaemia is only one of the many functions of the "antimegaloblastic substances" in the body, and their metabolic interrelationships are little understood. There can be no doubt of the importance of their role not only in nucleic acid metabolism but also in many fundamental processes in the body. Any one worker can only hope to find a few pieces to add to the metabolic jigsaw and in the present thesis there are described the results of certain investigations carried out in patients with megaloblastic anaemia of various types, and to a lesser extent in cases of malignant disease, in suitable controls, and in animals. The intention throughout has been to develop original lines of thought rather than to follow the work of others.

It is not yet possible to produce a final pattern of the metabolism in the body of the substances under consideration, but in the thesis an attempt has been made to consider present knowledge /

knowledge of the subject and to see to what extent our data may be fitted into the general scheme. The mechanism of production of the various forms of megaloblastic anaemia is considered, and there is put forward a more extensive classification than that usually employed.

Throughout this thesis the terminology employed for the bone marrow cells is that given in Whitby & Britton's "Disorders of the Blood" (7th Edition), and is based on that of Israëls. The name "intermediate erythroblast" or "transitional erythroblast" is given to cells that have characteristics intermediate between those of the normoblast and the megaloblast. The name intermediate megaloblast is given to the Ehrlich's megaloblast — the cell intermediate between the early and the late megaloblast.

The haemoglobin readings are calculated so that 14.8 G. per 100 ml. is equal to 100% on the Haldane scale. A conversion table is included at the end of the thesis.

The terminology used for bacteria is that commonly employed by workers in Britain engaged in the microbiological assay of haemopoietic factors. Thus L.leichmannii is used for Lactobacillus leichmannii, S.faecalis for Streptococcus faecalis and L.citrovorum for Leuconostoc citrovorum. The abbreviation B.coli is used rather than E.coli for Bacillus coli (Bact.coli, Escherichia coli).

Much of the information given in this thesis is presented in tabular form. As far as possible the tables have been simplified to enable them to be included on the standard page. A few tables, however, have had to be presented in a folded form/

form since they were too complex for satisfactory photographic reduction. The number of lines drawn in and around tables has been restricted to the minimum to avoid eyestrain to the reader.

A few colour prints of bone marrow cells are included, and for cooperation in the production of these the author is very much indebted to Mr. T.C. Dodds of the Department of Photomicrography, University of Edinburgh, Messrs. Gevaert of Belgium, and Messrs. Hamilton Tait of Edinburgh. Efforts to obtain such prints in Britain and the United States were unsuccessful, and results with the trichrome carbro process were quite unsatisfactory. Messrs. Gevaert made special arrangements to process the colour negatives in Antwerp. The colours are not yet entirely satisfactory but the pictures have been included because in most instances they give a reasonable indication of the morphology of the cells. The pictures are best examined in bright daylight. Possibly these photographs, made from negatives in the complementary colours, are the forerunners of inexpensive colour prints of blood and marrow cells and histological sections. In some instances black and white prints have been made from the colour negatives as keys to the parent illustrations.

The author is indebted to very many people for their interest, encouragement, advice, and assistance. In particular, he would like to mention Professor L.S.P.Davidson for his interest and advice throughout, Brigadier F.Harris (now Lieut.-General and Director General of the Army Medical Services) and Dr. A.M.Thomson (formerly Lieut.-Colonel, R.A.M.C.) for their encouragement in connection with the Indian/

Indian studies, Professor C.C.Sturgis and Professor F.H.Bethell for providing facilities at Ann Arbor, Michigan, Dr. M. E. Swendseid for her advice at Ann Arbor, Professor T.J.Mackie for providing facilities in the Department of Bacteriology at Edinburgh University, numerous physicians, surgeons and obstetricians in almost every hospital in the South-East of Scotland for providing access to patients under their charge, Mr. Robert Brown, technician in the Department of Medicine, University of Edinburgh, for assisting with many of the microbiological assays in the later part of the investigations, Dr. W.F.J.Cuthbertson of Glaxo Laboratories, Ltd., Dr. G.E. Shaw of Evans Medical House, Drs. E.L.R.Stokstad and H.Broquist of Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, Dr. A.T.Mennie of Lederle Laboratories Division, British Cyanamid Company, London, and Dr. Augustus Gibson of Messrs. Merck & Company, Rahway, New Jersey. These last helped a great deal by providing numerous drugs, chemicals and microbiological standards.

The illustrations on pages 116 and 117 are by courtesy of the Director of Public Relations, War Department, Government of India.

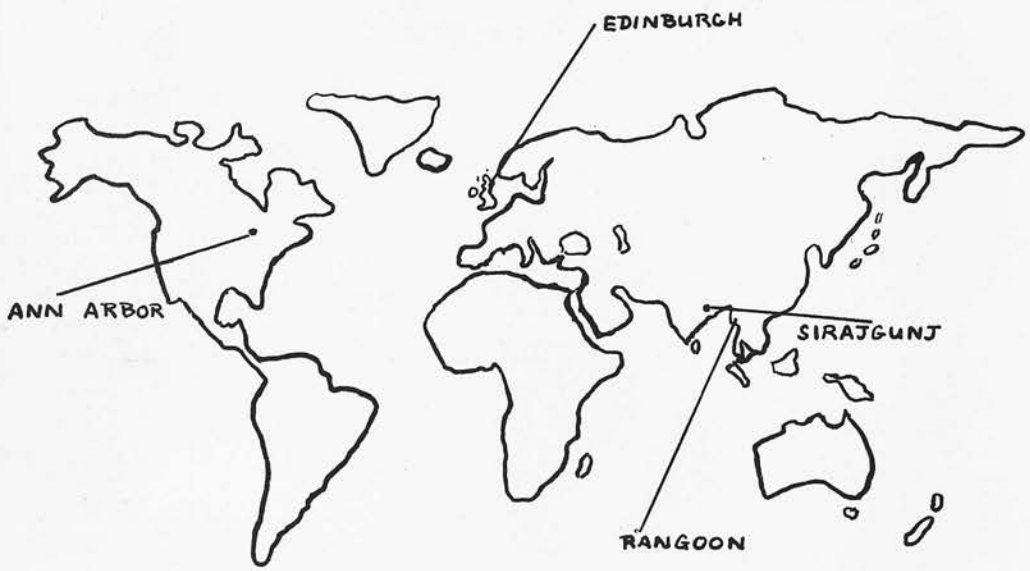
In particular the author would like to thank Sisters Cumming and Mackintosh of Edinburgh Royal Infirmary and, above all, the patients, for their co-operation.

CONTENTS

CHAPTER/

Clinical/

CHAPTER/



MAIN LOCI OF INVESTIGATIONS DESCRIBED IN THIS THESIS.

CHAPTER I

BRIEF HISTORICAL INTRODUCTION

The situation with regard to the substances with anti-megaloblastic activity and the various forms of megaloblastic anaemia is so complex that a short historical introduction is necessary to give perspective to the data that follow.

On May 1st, 1822, at a meeting of the Edinburgh Medico-Chirurgical Society, James Scarth Combe (1824) described a patient with a severe degree of anaemia which proved fatal in about six months. Although a post-mortem description is given, it is not possible to be certain that this was a case of pernicious anaemia. It is stated, for instance, that the tongue was covered with a dry fur; one cannot be certain of the diagnosis.

In 1855, Addison, describing the disease of the suprarenal glands which bears his name, wrote a few paragraphs on 'idiopathic anaemia', a condition arising from no discoverable cause. His description is essentially that of pernicious anaemia.

In 1874 an editorial in the Medical Times and Gazette contained an account of 'Pernicious Anaemia: A New Disease', described by Biermer of Zurich in 1872, but a week later Wilks (1874) reported that he had heard Addison lecture on the disease in 1843.

Thereafter there were published many papers dealing with pernicious anaemia. In 1877 Fenwick reported atrophy of the gastric mucous membrane: in 1883 Laache and in 1889 William Hunter described recurrent glossitis in the condition, and in 1897 Martius discussed the importance of achlorhydria, which had/

had, however, been recognised before that date. The high colour index was referred to by Laache (1883) and in 1901 William Hunter wrote a monograph on "Pernicious Anaemia: its Pathology, Septic Origin, Symptoms, Diagnosis and Treatment".

In 1875 Pepper described changes in the bone marrow in the disorder, and in 1884 Leichtenstern referred to neurological symptoms and signs in two patients, but thought that these were coincidental. Degenerative changes in the spinal cord were noted by Lichtheim (1887): the peripheral nervous features were reported in detail by Hamilton and Nixon (1921). Treatment by blood transfusion was mentioned by Pepper (1875) and in 1877 Byrom Bramwell drew attention to the value of arsenical therapy. It was in 1926, however, that Minot & Murphy, influenced by the researches of Whipple, Hooper & Robscheit-Robbins (1920) who had worked with dogs rendered anaemic by bleeding, showed that pernicious anaemia could be treated successfully by the feeding of 250-500 grammes of liver daily. Others had already tried dieto-therapy in pernicious anaemia. Gibson & Howard (1923) also influenced by Whipple's work, had fed a diet high in iron content and in vitamins, containing a certain amount of liver. They had not, however, recognised the importance of liver therapy.

There followed the commercial production of liver extracts for parenteral use, and about this time (1929) Castle published the first of a series of papers describing his brilliant experiments which led to the conclusion that the active principle of liver was formed by the interaction of an extrinsic factor in food and an intrinsic factor normally secreted by the stomach. In 1929 Sturgis and Isaacs found that the normal

hog's/

hog's stomach was also a possible source of a potent anti-pernicious anaemia substance. A comprehensive monograph on Pernicious Anaemia was published by Davidson & Gulland in 1930.

There the matter largely rested until the first apparently successful results of the treatment of pernicious anaemia with folic acid were announced in 1945, when this thesis was in its early stages of preparation.

In 1935 Day, Langston & Shukers had observed that an anaemia and severe leucopenia developed in young monkeys fed a diet lacking certain members of the B complex, and later absence of a special substance called vitamin M was postulated as the cause of the deficiency. It was then discovered by the same group of workers that a factor required for the growth of lactobacillus casei, the 'l.casei factor', would cure the anaemia in these monkeys.

In another field Stokstad & Manning (1938) had found that a diet containing all known essential dietary elements was lacking a growth factor for chicks, and the following year Hogan & Parrot claimed that such a diet led to the development of macrocytic anaemia in the chick. Pfiffner and his co-workers at the Parke, Davis Laboratories in Detroit, followed this up and isolated in 1943 the missing substance, then known as vitamin B_c. A conjugate was also isolated in crystalline form two years later.

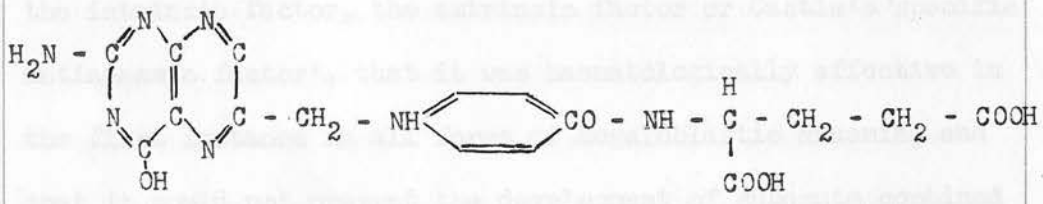
Snell & Peterson recognised in 1940 that certain lactic acid bacteria required extracts of plants or animal organs for satisfactory growth, and the following year Mitchell, Snell & Williams obtained from spinach a growth factor for Strepto-

occcus/

Streptococcus lactis R. To this was given the name of folic acid.

In 1945 Angier et al., working at Lederle Laboratories, Pearl River, New York, synthesised a substance which they claimed to be identical in its biological effects with the l. casei factor of liver, and in 1946 the same workers told of the chemical composition and method of synthesis of their factor. It was soon apparent that vitamin M, vitamin B_c, the liver l.casei factor, folic acid and the new synthetic substance were identical or closely related, and the name pteroylglutamic acid was given to the synthetic substance.

The formula is:



Pterin group PABA group Glutamic acid group

In/

In 1944 Sharp and his co-workers had tested the effect of vitamin B_c concentrate obtained from yeast on ten patients with pernicious anaemia. At the end of four weeks there was little evidence of improvement. It is now obvious that the dose had been too small.

In November 1945, Spies, Vilter, Koch & Caldwell gave the new synthetic folic acid intravenously to five patients with megalobastic anaemia and noted an immediate clinical and haematological response. Oral administration was also successful. The earliest part of the present investigations carried out in Britain concerned the clinical value of folic acid therapy in megaloblastic anaemia. This was commenced in April 1946. The work is now largely out of date and will not be reported in detail here.

It soon became obvious that pteroylglutamic acid was not the intrinsic factor, the extrinsic factor or Castle's 'specific antianaemic factor', that it was haematologically effective in the first instance in all forms of megaloblastic anaemia, and that it would not prevent the development of subacute combined degeneration of the cord in pernicious anaemia. (See Moore et al., 1945; Zuelzer & Ogden, 1946a,b; Garcia Lopez et al., 1946; Dalton et al., 1946; Davidson & Girdwood, 1946, 1947; Meyer, 1947; Peterson, 1947; Goldsmith, 1947).

Lester Smith (1948), working at the Glaxo Research Laboratories in England, succeeded as a result of many years of investigation, in isolating from liver another substance that was more potent than folic acid in its therapeutic effect in pernicious anaemia. He depended on clinical trial for assay of/

of the potency of his various preparations, but was able to make real progress because he had noted that the antianaemic activity followed a deep pink band on a partition chromatogram from refined liver extract. At the time when his discovery was announced, the preparation was amorphous, but very soon afterwards, this material was crystallised (Lester Smith & Parker, 1948).

Almost simultaneously with Lester Smith's first announcement, a group of workers in the laboratories of Messrs. Merck & Co. in the United States (Rickes et al., 1948a) announced the isolation from liver of a red crystalline material which they named vitamin B_{12}. The American team benefitted greatly from applying their knowledge of the work of Shorb (1947a,b, 1948).

In 1947 Shorb was attempting to find a microorganism that might require a rat growth factor found in liver extracts, certain caseins (Carey et al., 1946) and some foodstuffs (Hartman, 1946). She noticed that Lactobacillus lactis Dorner required the presence of two unidentified factors for growth in an amino acid basal medium containing all the synthetic B vitamins. One factor, present in tomato juice, may also be found in small amounts in casein and many other substances, while the other heat stable factor, referred to then as the L.L.D. factor, was found in higher concentrations in liver extracts active for rat growth, but not in casein or casein hydrolysates.

There emerged the extremely important finding that assays for L.L.D. factor in crude and refined liver extracts of the type used for the treatment of pernicious anaemia showed that the/

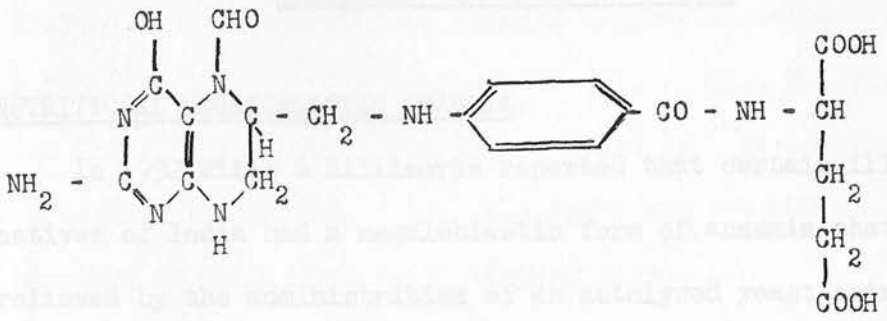
the L.L.D. factor effect in refined liver extracts was apparently proportional to the potency of these extracts for producing a satisfactory response in pernicious anaemia therapy. It therefore appeared possible that the L.L.D. factor might be the main active anti-anaemic principle in liver. What was, in fact, being measured was the vitamin B_{12} potency of the liver extract.

Microbiological assay methods for many vitamins, including pteroylglutamic acid, are relatively simple, but unfortunately such is not the case with vitamin B_{12}. In the present thesis there is given in summary form alternative methods of assay that we have developed in the course of our investigations.

Better, though incomplete understanding of the chemical nature of vitamin B_{12} has led to the use of the name cyanocobalamin for the substance.

In 1948 Sauberlich & Baumann described a growth factor for the streptococcus Leuconostoc citrovorum: this factor occurred in certain natural materials including liver, liver extracts and yeast extracts. It, too, has been found to be effective in the treatment of pernicious anaemia. Bond et al., (1949) prepared from liver a substance which they named folinic acid. The biological and clinical properties of citrovorum factor and folinic acid appeared to be similar, and the two substances are now believed to be identical; in 1950 Brockman et al. described the synthesis and isolation in crystalline form of citrovorum factor. The substance was shown by Pohland et al. (1951) to be 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid or

OH/



Some reference to its clinical value and the results of metabolic studies will be considered in later chapters.

In view of the fact that various authors use the terms in different ways, it should be stated here that in this thesis the terms citrovorum factor (CF) or folinic acid will be applied to those substances, natural and synthetic, that support the rapid growth of Leuconostoc citrovorum, a property not shown by pteroylglutamic acid (PGA). Streptococcus faecalis and Lactobacillus casei respond similarly to PGA and CF. The term folic acid will refer to these compounds, natural and synthetic, that support the growth of S.faecalis and L.casei but not of L.citrovorum. PGA will refer specifically to pteroylglutamic acid.

The main antimegaloblastic substances that have been isolated are those described above — cyanocobalamin, pteroylglutamic acid and citrovorum factor. There are, however, many others and these will be referred to later.

FORMS/

FORMS OF MEGALOBLASTIC ANAEMIA OTHER THAN ADDISONIAN PERNICIOUS ANAEMIA

NUTRITIONAL MEGALOBLASTIC ANAEMIA.

In 1932 Wills & Billimoria reported that certain ill-fed natives of India had a megaloblastic form of anaemia that was relieved by the administration of an autolysed yeast extract, and by crude, but not by refined liver extracts given by injection. This led to the hypothesis that, in addition to the main factor present in liver extracts there was another anti-megaloblastic substance, the Wills' factor. Macrocytic anaemia, which is not necessarily megaloblstic, is widespread in tropical climates, but the confusion in the literature is such that this form of anaemia will be considered in detail in a later section of this thesis.

INFESTATION WITH DIPHYLLOBOTHRIUM LATUM.

Incidence.

Infestation with the fish tapeworm is frequently associated with megaloblastic anaemia and this is most commonly seen in Finland. Infestation of humans with the worm does, however, occur in other countries, but it appears that in some of these the natives of the country do not suffer from an associated anaemia. This may be merely a reflection of the incidence of a proclivity to pernicious anaemia amongst the population exposed to worm infestation. The parasite itself is found to infest man especially in Finland, Upper Sweden, Russia, Esthonia, Latvia, Lithuania, Eastern Prussia, Switzerland and Japan. It has been reported in Denmark, Norway, Iceland, Poland, Russian Turkestan, the Netherlands, Belgium, France near the Swiss Border/

Border, Southern Bavaria, Austria, Hungary, Northern Italy, Ireland, the United States, Canada, Alaska, Siberia, Manchuria, Palestine, British Bechuanaland, Angola, Uganda, Madagascar, the Phillipines, Brazil and the West Indies. It has been calculated (Birkeland, 1932) that there are 500,000 to 750,000 carriers of this parasite in Finland alone.

The associated anaemia has been reported from Finland, Russia, Poland, Latvia, Lithuania, Germany, Switzerland and the Northern States of America. In America the condition has usually occurred amongst immigrants from the Baltic countries, but some cases have been reported amongst 'natives' of the country. Certain pike and perch in Lake Michigan are infested with the worm. A very few cases of diphyllobothrium latum anaemia have been reported from Sweden, Norway, Denmark, France, Austria, Italy and Japan. Three cases have been reported from Britain, two of the patients being Finnish (Harland et al., 1950; Stevenson, 1950).

Historical.

Even before 1883 Albrecht in St.Petersburg noted a close relationship between pernicious anaemia and infestation with the fish tapeworm. Botkin (1885) made the same observation and Hoffmann (1885) remarked that he had seen several cases of pernicious anaemia associated with diphyllobothrium latum infestation, in which anthelmintic treatment cured the anaemia. Many workers thereafter confirmed these observations.

The relationship between Addisonian pernicious anaemia and fish tapeworm infestation remained a mystery, and much research work was done. It was thought by many that the parasite produced a toxin which was absorbed into the blood stream, and various workers injected tapeworm extracts intravenously into experimental/

experimental animals. Schauman & Tallquist (1898) claimed to have produced 'hyperchromic anaemia' in dogs in this way. Tallquist (1907) appeared to produce temporary mild 'hyperchromic anaemia' in himself by taking by mouth a haemolytic lipoid extracted from the worms. It is of interest that he himself developed Addisonian pernicious anaemia twenty years later.

Seyderhelm (1918) prepared by repeated alcoholic precipitation from Diphyllobothrium latum a substance 'bothriocephalin' which, when injected into rabbits, was claimed by this worker and by Becker et al. (1925) to produce 'hyperchromic anaemia' with 'megaloblasts' in the marrow.

These and other similar experiments would appear to have been complicated by possible haemolytic effects of the extracts used.

Many persons harbour the fish tapeworm; few develop megaloblastic anaemia, and the development of the anaemia is not necessarily related to the 'load' of worms. There must, therefore be some predisposition or other factor affecting the host. It has been noted by several observers that some members of a family may have fish tapeworm anaemia, whilst others have Addisonian pernicious anaemia (e.g. Schauman, 1920).

The arrival of pteroylglutamic acid and cyanocobalamin on the scene has stimulated further investigative work, and recently it has been shown that the worm itself is a rich source of cyanocobalamin (see von Bonsdorff, 1948; von Bonsdorff & Gordon, 1951, 1952; Bjorkenheim, 1951). The likelihood is that in a predisposed person with histamine fast achlorhydria or hypochlorhydria, and associated deficiency of intrinsic factor,/

factor, the worms deprive the host of the cyanocobalamin of the diet. If it be true that the function of intrinsic factor is to promote the absorption of cyanocobalamin, then it can be understood that a diminished secretion of intrinsic factor may result in a diminished absorption of cyanocobalamin; it is not yet clear whether or not the worm ingests equally free cyanocobalamin and cyanocobalamin 'bound' to intrinsic factor. Indeed our knowledge of the normal action of intrinsic factor on cyanocobalamin is not yet complete.

MEGALOBLASTIC ANAEMIAS IN INFANTS AND CHILDREN.

At the time of writing, this rare group of megaloblastic anaemias requires further elucidation. It may be said, however, that in infants and children there are various forms of megaloblastic anaemia produced by different mechanisms. In 1944 Davis reviewed the literature and reported three children, respectively aged twelve, thirteen and fifteen years, who had megaloblastic anaemia. This was before the synthesis of pteroylglutamic acid or the use of the fat balance test, but it was noted that there was a response to proteolysed liver (which has a high content of folic acid) and it was suggested that 'defective assimilation' from the alimentary tract might be an aetiological factor of importance. Davis refers to twenty-one cases of macrocytic anaemia that had been reported in infants and sixteen cases in children. Sternal punctures had not been carried out in all of these, and in none could a diagnosis of true pernicious anaemia be made with certainty.

True pernicious anaemia in childhood is indeed extremely rare. Peterson & Dunn (1946) considered that the necessary diagnostic/

diagnostic criteria are:

1. Macrocytic anaemia.
2. Gastric achlorhydria resistant to histamine stimulation.
3. Megaloblastic "arrest" in the bone marrow.
4. Reticulocytosis following liver therapy.
5. Necessity of continuous therapy to maintain a continuous remission.

These authors, reviewing the literature to 1942, considered that most reported cases could be discarded — some were due to malnutrition, some patients had free hydrochloric acid in the gastric juice, some had haemolytic anaemia, one had Diphyllobothrium latum infestation, and one had staphylococcal septicaemia. In two cases the respective causes of the anaemia were congenital syphilis and Taenia saginata infestation. Presumably the anaemia was not megaloblastic in many of these patients.

Peterson & Dunn considered that in only two reported cases could a diagnosis of pernicious anaemia in childhood be made with certainty. These were in a girl of 13 years reported by Pohl (1940) and a female child of 13 months, reported by Dedichen (1942). These authors then reported a third case in a girl whom they had followed from the age of 13 months to the age of 5½ years. Fat balance tests were not done on these three patients, and it would now appear desirable in suspected cases of pernicious anaemia in childhood to carry out folic acid absorption tests of the type described in a later chapter of this thesis. In 1949 Hamilton & Fowler reported megaloblastic anaemia in a fourteen year old girl whose mother had achlorhydria and subacute combined degeneration of the cord. The child appears to fulfil all the "diagnostic criteria", but again a fat balance test was not done. In the same year Davis et/

et al. referred to a child of six years of age who responded in successive relapses to refined liver extract, pteroylglutamic acid and vitamin B_{12}. These authors claim that this child had true pernicious anaemia, but free hydrochloric acid was present in the gastric secretions. A fat balance test was not done. This case cannot be accepted as Addisonian pernicious anaemia. In coeliac disease, hypochromic anaemia is the type usually found, but megaloblastic anaemia may occur (e.g. Dalton et. al., 1946). No doubt some of the children reported to be suffering from pernicious anaemia have, in fact, had malabsorption of haemopoietic factors as a result of coeliac disease. Diphyllobothrium latum infestation must also be remembered as a possible cause.

In infancy the position is more complicated. Davis (1944) referred to the fact that some cases of macrocytic anaemia of infancy had occurred where the infants were on a diet of goat's milk, and Bass (1944) reported two cases of megaloblastic anaemia of infancy also associated with such a diet. In 1946 attention was drawn to the importance of megaloblastic anaemia of infancy by Zuelzer & Ogden in Detroit, Michigan, and by Amato in Italy, each of whom reported 25 cases. Zuelzer and his co-workers (1946a, b, 1947a, b) showed that the haematological features included not only macrocytic anaemia with a megaloblastic marrow, but also thrombocytopenia, leucopenia, and neutropenia with giant metamyelocytes and hypersegmented neutrophils in the peripheral blood.

The present author visited Zuelzer in Detroit in 1949 and saw some of the marrows. There was no doubt that megaloblasts were/

were present.

Megaloblastic anaemia of infancy appears to be essentially a condition of nutritional origin and it is permanently cured by suitable treatment. The difficulty is to explain the exact deficiency, and published reports since 1945 indicate that this probably differs in various patients. In the series reported by Zuelzer & Ogden (1946), two infants had been receiving only breast milk for a year. The mother of one had pernicious anaemia: the mother of the other had iron deficiency anaemia, glossitis and achlorhydria and her mother had pernicious anaemia. Another child had been fed goat's milk; six had clinical evidence of scurvy but most infants with scurvy did not have megaloblastic anaemia; seventeen had been receiving evaporated or dried milk. Pteroylglutamic acid was an effective therapeutic agent. At this point it might be advisable to digress in order to call attention to the findings of various authors for the folic acid content of milk. The most complete report of the amount of this vitamin in various substances is that published by the U.S.Department of Agriculture (1951). The investigation of the folic acid content of milk by this Department's own workers is not comprehensive but on a fresh weight basis, l.casei being used as the test organism, the reported content is:

Evaporated (canned) milk	0.7 µg./100 Gm.
Pasteurised milk	0.6 µg./100 Gm.

In this report the investigators have summarised all published figures, recalculating the results where the standard had been unsatisfactory. These data suggest that -

(a) in the samples taken, whole goat's milk contained approximately 0.12 µg./100 ml. (S.faecalis assay) when fresh,

but/

but this increased with storage at 4°C. to 0.86 µg./100 ml. after 7 days.

(b) whole cow's milk contains 0.02-2.2 µg./100 ml. depending upon the sample, the investigator and the details of the assay.

(c) human milk contains 0.14-10 µg./100 ml. depending upon the sample, the investigator and the details of the assay.

In other words the amount of folic acid in milk is very small, and the data available are too unsatisfactory for any conclusion to be drawn. Results of microbiological assays on natural substances containing only traces of the vitamins being investigated must be interpreted with great caution.

We are not therefore in a position to explain why goat's milk appears to produce in some infants a tendency to megaloblastic anaemia, but it appears possible that it is not due to any abnormality of its folic acid content. In fact associated ascorbic acid deficiency seems to be the likely explanation as far as the dietetic aspect of the question is concerned. More recent work has shown that some infants with megaloblastic anaemia respond to cyanocobalamin, whereas others do not respond to this but do improve with pteroylglutamic acid, and that there is some relationship between the metabolism of folic acid and of ascorbic acid.

In 1949 May et al. described two infants who had megaloblastic anaemia responding to injections of liver extract that gave a total of only 9 and 18 microgrammes of folic acid respectively. In the same year Luhby studied a number of infants who had megaloblastic anaemia responding to pteroylglutamic acid but not to cyanocobalamin, while Woodruff et al. described three infants who responded to the latter and two who did not.

The/

The important point that now emerged was that many of the infants with megaloblastic anaemia were receiving the same two proprietary dried milk preparation, and that the addition to these, not of pteroylglutamic acid but of ascorbic acid led to the virtual disappearance of megaloblastic anaemia of infancy in the United States. (May et al., 1950). The folic acid content of these preparations has not been published, but in any dried milk preparation it is small or even negligible (U.S. Department of Agriculture, Handbook No.29). As regards clinical response to therapy in megaloblastic anaemia of infancy the recent literature may be summarised as follows:

Response to Pteroylglutamic Acid.

Numerous reports have been given including those of Zuelzer & Ogden (1946a,b, 1947a,b); Blattner (1948); Siebenthal (1948); Luhby & Wheeler (1949); May et al. (1951).

Response to Cyanocobalamin.

Responses have been reported by McPherson et al. (1949), Woodruff et al. (1949) and Sturgeon & Carpenter (1950).

Response to Citrovorum Factor.

Woodruff et al. (1951) have described fraternal twins with a family history of pernicious anaemia (in the maternal grandfather) who both developed severe megaloblastic anaemia after infection and who responded to 75 µg. of citrovorum factor given daily by injection.

It must be said that the literature dealing with this subject is complex and that the various papers even by the same group of investigators betray much confusion of thought. It does appear -

(1) that megaloblastic anaemia of infancy is largely nutritional in origin and that the primary deficiency in/

in most instances appears to be of folic acid rather than vitamin B_{12}.

(2) that the mother does not necessarily show folic acid deficiency.

(3) that the affected infant must be born with diminished stores of folic acid since it is unlikely that a milk diet normally supplies much of the vitamin. If the milk was a main factor, megaloblastic anaemia would be very common in infants fed only milk for long periods. The synthesis of haemopoietic factors by bacteria in the infant's alimentary tract and the site of their absorption merits investigation.

(4) that ascorbic acid deficiency in the diet precipitates the megaloblastic anaemia in many, but that folic acid is the therapeutic agent required. Ascorbic acid is not effective.

(5) that prolonged infection and alimentary disturbances precipitate folic acid deficiency.

(6) that in some instances the deficiency is in cyanocobalamin. The points made in relation to folic acid in paragraphs (2) and (3) also apply in relation to cyanocobalamin.

The metabolic relationships between folic acid, citrovorum factor and ascorbic acid will be discussed in a later chapter. In conclusion it would appear sufficient to say that the infant requires folic acid, cyanocobalamin and ascrobic acid for normal metabolism and blood cell development and that the initial supply in the liver and elsewhere must be supplied by the maternal organism. As yet we have no knowledge of the importance, if any, of the intestinal bacteria of the infant as a source of haemopoietic factors to their host.

MEGALOBLASTIC ANAEMIA OF PREGNANCY AND THE PUERPERIUM.

During pregnancy megaloblastic anaemia may develop: sometimes this is not recognised until after the birth of the baby or termination of the pregnancy. It is obvious that the condition may occur where pregnancy complicates Addisonian pernicious anaemia, diphyllobothrium latum infestation,

intestinal/

intestinal malabsorption, severe malnutrition and certain abnormalities of the gastro-intestinal tract. Certain cases do not, however, appear to fit into any of these categories.

In 1842, Channing in Boston, Mass. reported ten fatal cases of anaemia associated with pregnancy, and three more were described by Lebert from Switzerland in 1854. Thereafter other workers writing on pernicious anaemia referred to cases occurring in association with childbirth. In 1919, however, Osler drew attention to the fact that permanent recovery frequently took place where this type of severe anaemia complicated pregnancy. The condition was thereafter recognised as something separate from pernicious anaemia.

Numerous other reports followed and in 1936 Heilbrun described the bone marrow findings. A series of 30 cases was described by Stevenson (1938) and in 1942 Davidson et al. pointed out that this type of pregnancy anaemia differed from Addisonian pernicious anaemia in that free hydrochloric acid was frequently present in the gastric juice and there was a lower frequency and degree of macrocytosis and ovalocytosis. Callender published an excellent critical review of the subject in 1944. It is now generally agreed that the colour index may be low in the condition, that macrocytosis may not be obvious in the peripheral blood, and that demonstration of megaloblastic change by examination of the bone marrow is usually necessary for diagnosis.

So far as treatment is concerned, this was unsatisfactory prior to the introduction of proteolysed liver and of synthetic folic acid. Sometimes there was a response to liver therapy but/

but many patients did not improve with this. The literature prior to 1944 in relation to therapy has been summarised by Callender (1944). In some cases death occurred even although transfusion was given, but frequently spontaneous remission occurred after childbirth. There is general agreement amongst various authors that megaloblastic anaemia may recur in a subsequent pregnancy.

The introduction by Davis et al. (1943) of proteolysed liver, a papain digest of whole liver, in the treatment of the megaloblastic anaemias was of particular value in relation to the therapy of this form of pregnancy anaemia. Proteolysed liver was life-saving in many such cases but the later introduction of synthetic folic acid as a therapeutic agent has led to the displacement of proteolysed liver as the treatment of choice. The main disadvantage of proteolysed liver is its unpleasant taste.

In 1945, Moore et al. reported the case of a multipara, aged 32 years, who developed macrocytic anaemia in each of five consecutive pregnancies. On the fifth occasion the anaemia was severe and the marrow megaloblastic; there was a prompt response to synthetic folic acid given intravenously. Details of the previous pregnancies were not available. Spies (1946) reported three cases treated in this way, but the first report from Britain was not until 1948, when Davidson, Girdwood and Clark wrote an account of successful folic acid therapy in three cases of megaloblastic anaemia of pregnancy and one case of Addisonian pernicious anaemia complicated by pregnancy. There have been no reports of megaloblastic anaemia/

anaemia of pregnancy failing to respond to folic acid.

In sharp contrast to the excellent results with folic acid therapy is the fact that there have been no reports of this type of anaemia in temperate climates responding to cyanocobalamin. Failure of vitamin B_{12} therapy was first reported by Bethell et al. (1948), and further cases have been published by Day et al. (1949), Furman et al. (1950), Ungley & Thompson (1950), Lederer (1951).

In tropical countries, on the other hand, it appears that cyanocobalamin has been effective in the treatment of some cases of megaloblastic anaemia of pregnancy. Kothari & Bhende (1950) and Patel & Kocher (1950) have had successful results from this form of treatment. The reason for this discrepancy would seem to be that the Indian workers are describing a nutritional megaloblastic anaemia complicated by pregnancy, whereas the workers in Britain and the United States are describing a different condition.

Kothari & Bhende (1950) and Bhende (1949) consider that there is no difference between nutritional megaloblastic anaemia and megaloblastic anaemia of pregnancy in temperate climates but there is little ground for such a belief. It is true that several authors have suggested that the condition occurring in pregnancy is of nutritional origin. Thus Bethell & Blecha (1942) reported from Ann Arbor that of 70 patients of low economic status admitted to their hospital maternity unit, 18 had macrocytic anaemia. Of 158 pregnant out-patients, mostly of low income groups, 27.2% had macrocytic anaemia, and there was a definite inverse relationship between the incidence of such anaemia and the animal protein content of the diet.

In/

In a study of pregnant women, carried out in rural areas, the figures were:-

Total Protein (Gm./day)	Number of Women	Per cent with Macrocytic Anaemia
80 and more	27	0
79 - 60	107	2.8
59 - 40	87	11.5
less than 40	13	30.8
Total	234	7.3

or, using the figures for animal protein:-

Animal Protein (Gm./day)	Number of Women	Per cent with Macrocytic Anaemia
50 and more	78	0
49 - 30	120	10.0
less than 30	36	13.9
Total	234	7.3

Sternal punctures were not carried out.

The above findings are reproduced in detail here since they show a surprisingly high incidence of macrocytic anaemia which is obviously related to the dietary intake of animal protein. It should be noted, however, that it is not possible to be sure to what extent the anaemia was megaloblastic and in most reported series of cases one of the features of megaloblastic anaemia of pregnancy has been that the colour index and mean corpuscular volume have frequently been low.

In summary, most of the known facts suggest that the megaloblastic anaemia of pregnancy reported from the tropics differs in its aetiology from most cases reported from countries with temperate climates. This will be referred to again in a chapter dealing with nutritional megaloblastic anaemia, and metabolic/

metabolic investigations designed to throw some light on the causation of the condition in Britain will be reported in a later chapter of the thesis.

MEGALOBLASTIC ANAEMIA ASSOCIATED WITH 'SPRUE'

Probably the first accurate description of tropical sprue was that given by Ketelaer in 1672. The name 'sprue' was introduced to this country by Manson (1880)as an anglicised version of a similar Dutch word. Manson defined the condition as one occurring in the tropics or subtropics or among persons who had previously resided in warm climates; the disease was one characterised by glossitis and stomatitis, by the passage of pale copious loose fermenting stools, and by flatulence, wasting and anaemia. The phrase 'the sprue syndrome' has been used in recent years to include not only the features found in tropical sprue, but also in idiopathic steatorrhoea and in coeliac disease. Since the introduction of the fat balance test of Cooke et al. (1946) it has been extended to include cases where there is megaloblastic anaemia and deficient absorption of fat but little other clinical evidence of intestinal malabsorption, and the term "malabsorption syndrome" has come into use.

It has long been known that anaemia is common in tropical sprue, and that the peripheral blood picture is commonly similar to that in pernicious anaemia. In 1929 Fairley et al. reported that in their 67 cases of tropical sprue the mean haemoglobin was 65.5%. In 49 patients the red cell count was less than 4,000,000 per c.mm. In 61.2% of the total patients thecolour index was unity or more, and in 19 of the remaining

26 it was from 0.8 to 0.99. The introduction of marrow biopsy as a routine method of investigation has shown that megaloblastic anaemia is indeed extremely common in tropical sprue. It is possible that 'tropical sprue' as described in South America and Puerto Rico is not always the same condition as that described by European authors, since primary malnutrition is so frequently a feature in the American cases. It may be noted, however, that Rodriguez-Molina, reporting on sprue in Puerto Rico in 1943 stated that a macrocytic form of anaemia with a megaloblastic marrow occurred in over 90% of cases.

In 1888 Samuel Gee described a condition of infantile steatorrhoea and subnutrition, and to this he gave the name of the "coeliac affection". It occurred principally in children between the ages of one and five years, but a somewhat similar clinical entity was noted to affect adults in non-tropical countries, and in 1929 Holmes & Starr gave it the name of "non-tropical sprue". In 1932 Bennett et al. used the term idiopathic steatorrhoea for the condition in children and adults.

It is true that in certain cases of adult idiopathic steatorrhoea, coeliac disease has been present in childhood but a history of coeliac disease is by no means always obtained. For this reason it is better not to prejudge the question of a common aetiology by applying the same name to the forms in the child and in the adult. In idiopathic steatorrhoea in the adult, megaloblastic anaemia frequently occurs, whereas in coeliac disease of childhood iron deficiency anaemia is the usual type to be found. Thaysen (1931, 1935) reported "hyperchromic"/

"hyperchromic" anaemia in most of his adult cases and hypochromic anaemia in children.

It is well recognised that lacteal obstruction by tuberculosis of the mesenteric glands or from other causes may give rise to megaloblastic anaemia. In 1923 Ryle described a case with the features of idiopathic steatorrhoea due, in fact, to caseous tuberculous glands in the mesentery.

Anaemia in tropical sprue is bound up with nutritional megaloblastic anaemia and will be referred to again under that heading. A later chapter will deal with changes in the metabolism of folic acid in the sprue syndrome. Response to treatment with pteroylglutamic acid, cyanocobalamin and citrovorum factor will also be considered.

MEGALOBLASTIC ANAEMIA ASSOCIATED WITH GASTRO-INTESTINAL DISEASE OTHER THAN TROPICAL SPRUE, IDIOPATHIC STEATORRHOEA, COELIAC DISEASE OR LACTEAL OBSTRUCTION.

Megaloblastic Anaemia associated with Stomach Operations or other Gastric Abnormalities.

Many papers have dealt with this subject, but there is a distinct discrepancy in the views of various authors about the incidence of a "pernicious anaemia like condition" after total or partial gastrectomy. The chief reason for this is that most of the cases occurred before the routine employment of marrow puncture as an aid to diagnosis.

Total Gastrectomy. MacDonald et al. (1947) critically reviewed the literature dealing with patients who had survived total gastrectomy for three or more years. The total number of patients was forty-six; twelve of these had macrocytic anaemia./

anaemia. In two the marrow was megaloblastic, in one it was not, and in the others marrow biopsy had not been done. In a further five poorly documented cases of the series macrocytic anaemia may have been present. Since the data for the exact haematological pictures are so scanty it is not possible to say much about the response to treatment, but it does appear that several cases of macrocytic anaemia have responded to parenteral liver therapy and five have responded to treatment with pteroyl-glutamic acid (those of Ransom, quoted by MacDonald et al. 1947; Smithwick, 1947; Zollinger & Finch, 1947 also quoted by MacDonald et al.; two cases of Chados & Ross, 1951). One of the cases referred to by Chados & Ross is of interest in that subacute combined degeneration of the cord developed during pteroylglutamic acid therapy and this complication responded to the parenteral administration of liver. In contrast Beebe & Meneely (1949) have described a patient who had a total gastrectomy operation for syphilitic gastritis and who developed a severe macrocytic anaemia and subacute combined degeneration of the cord five to eight years later without having received any pteroylglutamic acid.

The best documented case of the whole series is that of Meyer et al. (1941) who describe a patient who had a total gastrectomy operation for linitis plastica. He was seen six years after gastrectomy and was found to have macrocytic anaemia with a megaloblastic marrow. The marrow was converted to the normoblastic state by parenteral liver therapy and there followed a rise in the blood counts but the patient thereupon committed suicide!

Macdonald/

Macdonald and his colleagues share the view of Sturgis & Goldhamer (1939) that it takes two or more years for macrocytic anaemia to develop after the operation of total gastrectomy, and consider that megaloblastic anaemia will develop if the patients live long enough. Sturgis & Goldhamer considered that this was because of the ability of the liver to store the 'erythrocyte maturation factor'. Another possibility for which there is as yet little experimental evidence is that some intrinsic factor is secreted in the duodenum or small intestine of man.

Partial Gastrectomy. Following partial gastrectomy macrocytic anaemia has been reported by many workers. Again the data are incomplete, but there is no doubt that true megaloblastic anaemia may occur, and Rhodes & Greenberg (1943) have described a patient who developed megaloblastic anaemia as long as fifteen years after partial gastrectomy for gastric ulcer. This patient improved with parenteral liver therapy. A case that showed a response to cyanocobalamin has been recorded by Conway & Conway (1951). The fundal region of the stomach is the principal part that secretes intrinsic factor and it is probably for this reason that megaloblastic anaemia after partial gastrectomy is not more commonly seen.

Carcinoma of the Stomach. It is frequently stated that macrocytic anaemia may develop in gastric carcinoma. On the other hand it is generally believed that there is an increased proclivity to gastric carcinoma in those who have pernicious anaemia. Sturgis & Goldhamer (1939) described a patient with macrocytic anaemia in association with gastric carcinoma who showed a remarkable response to a dessicated stomach preparation and/

and Beyers et al. (1952) have published a report of a patient with linitis plastica, possibly due to sarcoidosis, who developed true megaloblastic anaemia. The blood responded only partially to pteroylglutamic acid by mouth, but improved satisfactorily following the parenteral administration of cyanocobalamin. Oppenheim et al. (1945) have analysed 122 cases of gastric carcinoma and have shown that 25% had macrocytosis. This was not, however, associated with other peripheral blood features to suggest pernicious anaemia, and there was no response to liver extract. These workers consider that the anaemia is associated with hepatic insufficiency.

Megaloblastic Anaemia associated with Abnormalities of the Intestinal Tract.

The reported lesions of the intestinal tract associated with macrocytic anaemia are many, but again there is the difficulty that megaloblasts have not been demonstrated in most instances. A review of the literature to date was given by Butt & Watkins in 1936. In 1939 Barker & Hummel reviewed 49 published cases and recorded two others, and in 1949 Cameron, Watson & Witts again analysed the literature. A few further cases have since been published.

The lesions that have been reported in association with macrocytic anaemia are as follows:

Strictures

Small intestine

Tuberculous
Non-tuberculous, including regional ileitis

Large intestine

Tuberculous/

Tuberculous
Non-tuberculous, including carcinomatous

Anastomoses:

Entero-enterostomy
Entero-colostomy
Gastro-jejuno-colic fistula
Gastro-colic fistula
Gastro-enterostomy

Diverticulosis

Tuberculous adenitis of mesentery

Intestinal lipodystrophy

Regional ileitis

Abdominal lymphogranuloma maligna

Abdominal lymphangioma cysticum mesenterica, or chylangioma

Regional jejunitis

The last three rare causes were described by Bjerkelund (1950).

When one searches for records of bone marrow findings even in the more recently described cases, one is faced with the difficulty of judging what each author means by the term 'megaloblast', and in most instances no marrow findings are available.

At the time of writing, megaloblastic anaemia, rather than merely macrocytic anaemia, has been reported in eleven such patients.*

Tuberculous ulcer and stricture of the lower ileum (Zadek, 1926).
Fibrous tuberculous ulcer of the transverse colon (Zadek, 1926)
Foreign body in abdominal cavity with intestinal anastomosis and blind cul-de-sac (Hartmann, 1929).
Gastro-jejuno-colic fistula (Fairley & Kilner, 1931).
Tuberculosis of small intestine with multiple strictures (Hawksley & Meulengracht, 1936).
Ileo-transverse colostomy with blind loop (Barker & Hummel, 1939).
Gastro-jejuno-colic/

*A paper published later is referred to on p. 528.

Gastro-jejuno-colic fistula (Barker & Hummel, 1939).
Entero-enterostomy for regional ileitis (Cameron, Watson & Witts, 1949).
Intestinal lipodystrophy (Jones, Darby & Totter, 1949).
Regional jejunitis (Bjerkelund, 1950).
Chylangioma mesenterica (Hotz & Zollinger, 1942).

At this stage detailed consideration of the mechanism of production of megaloblastic anaemia in the various gastro-intestinal conditions would serve little purpose. The simplest explanation would be that after total gastrectomy, in some cases of partial gastrectomy, and in extensive gastric carcinoma the source of intrinsic factor is removed. It is easy to understand how extensive intra-abdominal disease may cause deficient absorption of cyanocobalamin and of folic acid from obstruction of the lacteals and mesenteric glands. Removal of a large segment of the small intestine, or by-passing of much of the small intestine from a gastro-colic or entero-colic fistula will also interfere with absorption. In addition, however, the possibility of destruction of haemopoietic factors by intestinal microorganisms, especially in blind intestinal loops, requires consideration. Reference to this will be made in later chapters. It is also not impossible that toxins may be be found in the intestinal tract in some of the conditions under consideration and that these may be absorbed and may subsequently interfere with the metabolism of the antimegaloblastic substances.

MEGALOBLASTIC ANAEMIA IN LIVER DISEASE.

In portal cirrhosis of the liver macrocytic anaemia is very common, but the lack of detailed haematological reports and confusion of different haematological terminologies makes it/

it difficult to say which authors are referring to true megaloblastic anaemia in their papers.

There is no doubt that the liver is an important storage organ for cyanocobalamin and for folic acid or citrovorum factor, but the kidneys also contain relatively large quantities of these as will be described in a later chapter. Nevertheless it is reasonable to consider the likelihood that longstanding hepatic cirrhosis may interfere with the metabolism of haemopoietic factors. Goldhamer et al. (1934) reported that the liver of a patient with macrocytic anaemia who had died of hepatic cirrhosis did not contain haemopoietic factors as measured by its ability to cause a remission in pernicious anaemia patients, whereas Schiff et al. (1938) using a similar technique considered haemopoietic factors to be present in the livers from two patients with portal cirrhosis, one with biliary cirrhosis secondary to carcinoma of the bile duct, one with chronic passive congestion of the liver and one with leukaemic infiltration of the liver. The graphs published by Schiff and his colleagues to show the haematological response in pernicious anaemia following the parenteral administration of extracts of these livers are certainly convincing. The method used to prepare the extracts was similar to that employed to isolate Cohn's Fraction G.; this method was used by most manufacturers as the basis for the preparation of liver extracts for therapeutic purposes in the days preceding microbiological and chromatographic techniques.

> 500 grammes of the liver obtained at post mortem was finely chopped in a litre of water and stirred. It was heated on a steam bath to 80°C. until the protein was/

was precipitated. After filtration, enough concentrated hydrochloric acid was added to the filtrate to precipitate acid-coagulable protein. After further filtration the filtrate was concentrated under reduced pressure to 300 ml. and enough 95% alcohol added to make the solution 70% alcohol. After filtration the alcohol was removed under reduced pressure. The remaining fluid was concentrated to 100 ml. and filtered.

The amounts injected into pernicious anaemia patients were:

From the first cirrhotic liver 10 ml. (equal to 50 G.) on two successive days.

From the second cirrhotic liver 20 ml. (100 G.) once.

From the liver of biliary cirrhosis 10 ml. (50 G.) once.

From the congested liver 20 ml. (100 G.) once.

From the leukaemic liver 10 ml. (50 G.) on two successive days.

A haematological response occurred in all instances. Reference will be made to this again in the chapter dealing with assays of haemopoietic factors in the human liver. (Page 262).

Berman et al. (1949) in a detailed consideration of the haematological state in patients with hepatic cirrhosis consider that megaloblastic anaemia does not occur, and this view is shared by Meulengracht & Gormsen (1948) and by many others. Jarrold & Vilter (1949) describing the blood and marrow pictures in thirty patients with chronic hepatic insufficiency stated that megaloblasts or early erythroblasts were present in three, but their terminology is not clear. On the other hand Movitt (1950) states quite categorically that he has encountered three patients with megaloblastic bone marrows in association with cirrhosis of the liver, the megaloblasts being indistinguishable from those of pernicious anaemia. Free hydrochloric/

hydrochloric acid was present in the gastric secretions of each patient. One responded well to an injection of 150 microgrammes of cyanocobalamin and the other two improved with parenteral liver therapy.

MEGALOBLASTIC ANAEMIA IN OTHER CONDITIONS.

Megaloblastic change is occasionally found in the marrow in leukaemia especially following therapy with aminopterin. Folic acid metabolism in relation to malignant disease forms the subject of a later chapter. Chapter 16 deals with "Refractory" Megaloblastic Anaemia.

CHAPTER 2.

NUTRITIONAL MEGALOBLASTIC ANAEMIA: REVIEW OF THE CONDITION, PARTICULARLY IN INDIA, AND DESCRIPTION OF CLINICAL INVESTIGATIONS CARRIED OUT IN INDIA DURING THE 1939 - 45 WAR

In most sections of this thesis a detailed summary of literature has not been given since satisfactory reviews are already available. Such is not the case with nutritional megaloblastic anaemia. The literature is much confused and there is lacking a consolidated record of the background upon which megaloblastic anaemia is superimposed in the tropics, especially in India and Pakistan. It is for this reason that the present account is offered, and, as will be seen, it is not possible to omit iron deficiency anaemia entirely from consideration.

* * * * * * * * * * * * *

Megaloblastic anaemia was,unexpectedly, a serious problem amongst Indian troops in the fighting areas during the campaign against the Japanese, and the term 'malnutrition' was at first used in discussion of the problem amongst the Army authorities. This seemed to the present author, who was given the task of defining the type of anaemia present, to be a most unfortunate name. Not only did it prejudge any decision about the cause of the syndrome, but general use of the term 'malnutrition' would have given the enemy the gift of a propaganda weapon which he would not hesitate to employ to lower the morale of our Indian troops. For this reason the name/

name 'marasmus syndrome' was suggested and universally adopted in discussion of the condition.

Conditions in the field were such that it was only possible to sketch out the problem. Further investigations were planned, but the collapse of the war in the East resulted in the cancellation by the Army authorities of the projected research work. It is, however, now obvious that microbiological assay methods for the measurement of haemopoietic factors, involving the use of techniques then unknown, would have been required.

It should be stated at once that the reason for the development of megaloblastic anaemia amongst large numbers of troops east of the Brahmaputra river was not altogether obvious and that even the most experienced of observers were uncertain whether the condition was due to primary malnutrition, tropical sprue, some other unknown cause, or a combination of factors. The background of the condition is considered in the following pages.

THE BLOOD FIGURES IN THE HEALTHY NORMAL WELL NOURISHED INDIAN.

Males. Napier & Das Gupta (1935, 1936) have given the mean haemoglobin levels and red cell counts for two series of 50 and 30 healthy Indian males, of good families, as being respectively 14.77 G./100 ml. and 15.7 G./ 100 ml., and 5,362,000 and 5,533,000 per c.mm. Sokhey et al. (1937) gave the mean figures for 121 healthy young men in Bombay, most of whom were University students, as Hb. 15.37 G. per 100 ml., (range 12.75 - 17.61); red cells 5,111,000 (range 4,070,000 - 5,950,000) per c.mm.; PCV 41.72% (range 32.47 -49.02%). The haemoglobin/

haemoglobin estimations were done by the oxygen capacity method of van Slyke & Neil (1924), Huffner's factor being used. The mean corpuscular volumes and mean corpuscular haemoglobin concentrations were not calculated but in any case the venous blood was collected in potassium oxalate and no allowance was made for cell shrinkage in the calculation of the packed cell volumes. In 1938 Sankaran & Rajagopal, also using van Slyke & Neill's method, calculated the mean haemoglobin value of 125 healthy males, mostly students and doctors, in South India to be 16.57 G. per 100 ml. The ages ranged from 18 to 25 years.

Non-pregnant females. Sokhey et al. (1938) also made a survey of the haematological findings in 101 healthy Indian women. These were medical students or nurses, aged 16 to 30 years. The mean figures were Hb. 12.99 G. per 100 ml. (range 10.46 - 16.01), red cells 4,465,000 (range 3,500,000 - 5,350,000) per c.mm.; PCV 36.27% (range 28.40 - 44.67%). Sankaran & Rajagopal (1938a,b) gave the mean haemoglobin level of 62 female students and doctors aged 17 to 22 years as being 13.73 G. per 100 ml. In their second paper they made the surprising claim that when six grains of ferrous sulphate was given daily for four and six weeks respectively to two separate groups of 24 girls the mean haemoglobin level rose in the groups from 16.35 G. per 100 ml. to 20.34 G. per 100 ml. and from 13.69 G. per 100 ml. to 18.44 G. per 100 ml. Napier & Edwards (1941) gave the mean figures for 128 Calcutta women aged 14 to 38 years as haemoglobin 91.5% red cells 4,615,000 per c.mm.; MCV 86.8 cμ., MCHC 31.6%.

Pregnant Females. In 64 'normal' pregnant females at varying stages of the second half of pregnancy, Napier and Edwards (1941) found the mean haemoglobin level to be 78.2%, red/

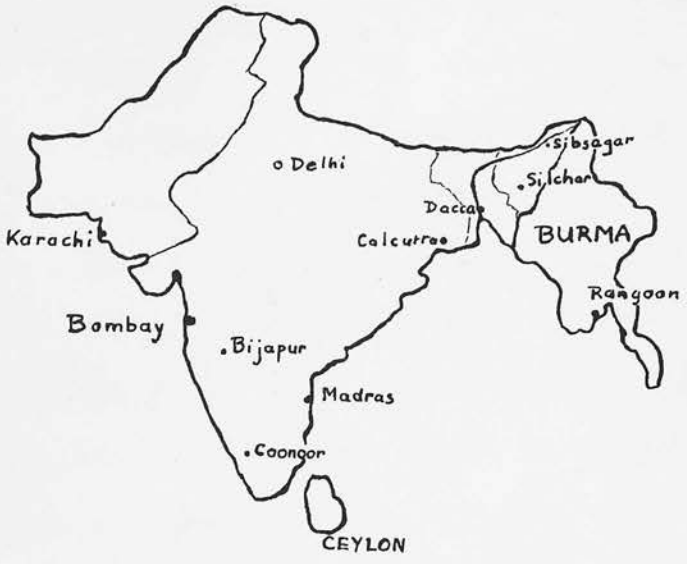
red cells 4,120,000 per c.mm., MCV 86.8 cμ., MCHC 30.6%. There was no significant increase in the mean corpuscular volume with the lengthening of the duration of pregnancy. Radhakrishna Rao (1938) has stated that in Coonoor in South India, at an altitude of 6,000 feet, the mean haemoglobin level for 100 pregnant females was 15.52 G. per 100 ml. and for 100 non-pregnant females it was 15.81 G. per 100 ml. The difference was not significant. In relation to this it should be noted that Sankaran & Rajagopal (1938c) have reported that the mean haemoglobin level of thirty-eight British soldiers sent to Coonoor rose from 12.84 to 19.56 G. per 100 ml. in three weeks. No further rise occurred thereafter.

The figures given by Ghosh et al. (1948) for some 1,000 patients during pregnancy or in the sixth to eighth postnatal weeks in Calcutta are of particular interest since the instruments used were more satisfactorily standardised than those of some of the other workers. In pregnancy the mean figures were Hb. 10.51 G. per 100 ml., red cells 4,170,000 per c.mm., MCV 81.3 cμ., MCHC 31.61%. In the postnatal group they were Hb. 10.77 G. per 100 ml., red cells 4,420,000 per c.mm.,MCV 80.37 cμ., MCHC 30.44%. These are selected patients in that they were in or attending hospital, and therefore of fairly good social class.

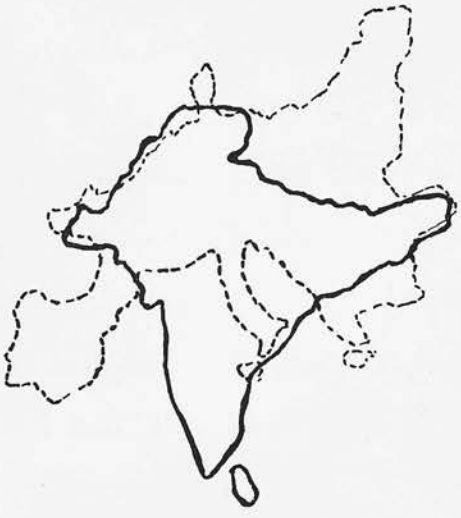
Enough has been said to show that there is no material difference between the normal blood figures for the relatively well-to-do Indian and the European.

ANAEMIA IN THE POORER INDIAN.

In/



MAP 1. INDIA AND PAKISTAN.



INDIA IS SO VAST THAT GENERALISATIONS SHOULD BE MADE WITH CAUTION.

Maps of India and Continental Europe superimposed on same scale.

In 1939 Macdonald, using a Talquist haemoglobinometer, carried out approximately 10,000 haemoglobin estimations on labourers in a tea estate in the Sibsagar district of Assam. The estate was at an altitude of 500 feet and was situated near the Brahmaputra river (See Map 1.).

The Talquist haemoglobinometer is inaccurate but Macdonald considered that the great number of estimations compensated to a large extent for this inaccuracy. His findings were as shown in Table 1.

	Number of estimations	Mean Hb. (%)	Hb. Range (%)	Standard Deviation
Men	2,826	70.09	20 - 90	7.28
Women	2,663	65.03	20 - 85	9.69
Children	3,553	66.31	20 - 85	8.14

Table 1. Haemoglobin Survey of Tea Garden Coolies (Macdonald, 1939).

His figures for the vital statistics and morbidity in the area, with a population of approximately 10,000, are of interest.

Death Rate	31.2
Birth Rate	43.0
Infant Mortality Rate	169

Macdonald considered that no reliable Indian figures for vital statistics were available for comparison.

Calculated over a four year period the chief causes of death were:

Chest diseases	7.06 per 1,000 per annum.
Alimentary diseases	4.75 per 1,000 per annum.
Malaria	3.03 per 1,000 per annum.
Anaemia	2.98 per 1,000 per annum.
Childbirth	1.14 per 1,000 per annum.

The/

The morbidity caused by anaemia was estimated by Macdonald as being more than 3,000 working days lost per 1,000 persons per annum; the incidence of anaemia varied in different estates.

Anaemia in Indian labourers is by no means limited to Assam, but indeed occurs throughout India. Hare (1940) found that recruits to Assam tea estates from various parts of India did not differ from the local coolies in this respect. Europeans on the estates were not anaemic.

Napier & Billimoria (1937) analysed the blood findings of fifty-two anaemic pregnant women on a tea estate and grouped their results according to the mean corpuscular haemoglobin. The distribution was:

Hyperchromic, with severe anaemia	(Hb. 2.75-5.77 G./100 ml.)	8 cases
Hyperchromic, with slight anaemia	(Hb. 7.97-10.31 G./100 ml.)	10 cases
Orthochromic, with moderate anaemia	(Hb. 6.18-8.25 G./100 ml.)	6 cases
Hypochromic	(Hb. 2.47-9.07 G./100 ml.)	28 cases

Napier & Majumdar (1938), in a further survey first gave blood findings for 25 male and 25 female working coolies aged 20 to 40 years in tea gardens near Silchar. The results are summarised in Table 2. (See Map 1.)

In pregnancy, anaemia was rife, but the coolies were fatalistic and would be found working with haemoglobin levels of 3 G. per 100 ml. or less.

In 57 cases of anaemia of pregnancy the distribution, based by the authors on a normal MCH of 22-33 yy and MCV of 75-/

TABLE 2.

Blood Findings in 25 male and 25 female teagarden Coolies
(Napier & Majumdar, 1938)

		Range	Mean
Hg. (G. per 100 ml.)	Males	7.0-15.5	12.6
	Females	6.9-13.3	10.4
Rbc (Mills./c.mm.)	Males	3.9-6.1	5.1
	Females	3.0-5.5	4.5
PCV (%)	Males	28.3-51.2	42.2
	Females	18.0-47.6	37.1
MCV (c.μ.)	Males	60.1-105.5	84.9
	Females	56.0-108.0	82.5
MCHC (%)	Males	26.9-39.3	29.7
	Females	21.7-38.3	28.7

75-105 c.μ. was:

Hyperchromic macrocytic anaemia	3
Orthochromic orthocytic anaemia	23
macrocytic anaemia	5
Hypochromic microcytic anaemia	7
orthocytic anaemia	18
macrocytic anaemia	1

It should be noted that there is some doubt about the accuracy of the speed of the centrifuge used in this and many other Indian surveys.

In 1941 Napier & Edwards published a long report in the Indian Medical Research Memoir series dealing with Anaemia in Pregnancy in Calcutta. In 465 pregnant women selected as being anaemic, the relationship between the haemoglobin level and the mean corpuscular volume was as shown in Table 3.

TABLE 3.

Relationship between haemoglobin level and mean corpuscular volume in pregnancy anaemia (Napier & Edwards, 1941)

Hb.%	10-50	51-67	> 68
MCV (cμ.)	100.7	89.5	90.5
No. of Cases	154	147	162

The lowest MCHC findings were in the most anaemic patients. Severe anaemia was relatively more common in the 20 to 30 year old patients than in those who were younger or older. Recently Ramalingaswami & Patwardhan (1949) estimating the haemoglobins of 338 South Indian labourers of both sexes, found the haemoglobin to be below 13.1 G. per 100 ml. in 291 of them.

The general conclusions from a reading of these and other papers/

papers would seem to be that the normal haematological standards of well-nourished Indians, are similar to those of Europeans and this is so in males and in females. Unfortunately there are vast numbers of Indians who are ill-nourished and riddled with disease and amongst them anaemia is very common. This is so in both sexes, and the anaemia is found especially in pregnancy. Hypochromic anaemia is the type most frequently seen, but a raised mean corpuscular volume may be found in the anaemia both of males and of females. Macrocytic anaemia occurs especially in pregnancy. Apart from obvious cases of nutritional megaloblastic anaemia as discussed later, reports of marrow findings are very few.

CAUSES OF THE ANAEMIA OF INDIAN WORKERS.

Hookworm infestation.

Ankylostomiasis is common in India, but many investigators consider that this disease is not itself sufficient to explain the severe anaemia of many Indian labourers, even when it is hypochromic and microcytic. Macdonald (1939) drew attention to the lack of any relationship between the degree of hookworm infestation and the haemoglobin level. Deworming alone would not cure the anaemia. Napier & Billimoria (1937) found that the hookworm load was heavier in the pregnant tea garden coolies with hypochromic anaemia than in those with other types of anaemia, but 82% of their non-anaemic control patients had ova of ankylostoma in their stools. However, in the hypochromic group the importance of hookworm infestation could not be overlooked. Ramalingwasami & Patwardhan (1949) attribute the anaemia of South Indian labourers to hookworm infestation.

Malaria./

Malaria.

In Macdonald's investigation of 10,000 labourers, the spleen rate in the years of the investigation varied from 26.2% to 51.0%. Macdonald was convinced that malaria was not the main cause of the anaemia that was so prevalent but he did find that anaemia was most frequent in newly recruited labourers and in settled workers on estates with a high proportion of newly recruited labour. He wondered whether this might indicate that infection with a fresh strain of malaria upset the balance of infection and immunity and added to the anaemia in this way. He treated 42 anaemic women, who had haemoglobin readings between 35% and 79% with atebrin injections and iron by mouth. There was a significantly greater increase in the haemoglobin levels in this group compared with women receiving only iron. In general most workers have tended to minimise the importance of malaria in the causation of anaemia in Indian labourers. Obviously, though, it must play some part.

Diet.

Here we are on difficult ground, and it may be said from the commencement that the only way to gain satisfactory information would be for a team of investigators to spend a period of time with coolies in the areas under survey. These investigators would require to obtain duplicate samples of the meals consumed by the coolies, cooked in exactly the same way and to estimate in these foodstuffs the amount of iron, protein, ascorbic acid, folic acid and cobalamin compounds. Some information may be obtained from tables giving the values for these/

these substances in various foodstuffs, but this does not allow for variation in local cooking methods and their effects on the content of haemopoietic factors. An investigation of this sort has not yet been carried out, and it was not and could not be done in the jungles during the anti-Japanese operations or in the Japanese P.O.W. camps. For this reason our knowledge of the aetiology of the anaemia encountered during the war must always remain fragmentary.

In their account of the blood findings in 52 anaemic pregnant coolies, Napier & Billimoria (1937) stated that the intake was about 2,200 calories per day. Meat was eaten about once a week, little milk or eggs was taken, and the diet consisted chiefly of rice, dhal (a pulse) and vegetables. Napier & Majumdar (1938), again investigating coolie women, found that the total number of calories eaten was relatively satisfactory, but that protein was low, animal protein being negligible. About 12 G. of fat was taken daily. If all the iron taken was available the intake was adequate in this respect, but the diet was deficient in calcium, vitamin C, vitamin A and the B complex. No detailed information is given.

Macdonald (1939) in the course of his large haemoglobin survey gives figures for the diet consumed by three typical families. These are shown in Table 4.

The diet is seen to have a high content of iron, but to be deficient in animal protein, calcium and vitamins B_1 and B_2. Vitamin A and carotene are also lacking. It should be noted that the values for the various constituents of the diet were obtained from tables and that the problem of the availability of the/

TABLE 4.

Specimen Diets of Three Coolie Families (Macdonald, 1939).

Diet in grammes per man per day

Family	Rice	Dhal	Oil	Onion	Green Vegetables	Potato	Meat	Fish
I	800	44	4.6	0.3	41	20	-	3.2
II	795	66	10.0	7.6	46	-	66	-
III	765	27	8.6	12.5	64	33	69	-

Analysis of these diets (Daily intake)

Family	I	II	III
Calories	2514	3066	2914
Carbohydrate (G.)	549	659	627
Protein			
Vegetable (G.)	68	74	73.3
Animal (G.)	0.5	22.2	15.7
Fat (G.)	8.4	16.4	14.7
Iron (mg.)	24.3	30.4	26.6
Calcium (mg.)	0.22	0.20	0.15
Vitamin B_1 (I.U.)	789	979	900
B_2	present	present	present
C (mg.)	24	28	30
Carotene (Int.Vit.A Units)	1162	1442	1040
Vitamin A (Int.Vit.A Units)	1.0	31	41

the iron remains. Macdonald seems to be calculating total iron rather than available iron.

Macdonald also examined four groups of 69 women. In each group the diet was similar except for the type of rice eaten. The mean haemoglobin levels of each of the four groups were as shown in Table 5.

TABLE 5.

Influence of type of Rice eaten on the haemoglobin level in four groups of Coolie Women (Macdonald, 1939).

Group	Type of Rice	Mean Haemoglobin Reading (Hellige 100= 13.67g.%)
A	Home pounded parboiled	75.93%
B	Home pounded raw	71.72%
C	Milled raw	70.77%
D	Milled parboiled	67.10%

It should be noted that the instrument used for this part of the investigation was the Hellige haemoglobinometer. The difference between the readings in Groups A and C are significant, and between A and D are highly significant. This superiority of home pounded parboiled rice was also found from an analysis of haemoglobin readings in a much larger number of coolies whose blood was estimated on the Talquist scale.

Milling the rice would therefore appear to increase the incidence of anaemia.

It will be recollected that parboiling rice consists of steaming the paddy (rice in its husk) after preliminary soaking so/

so that the outer husk splits and becomes easier to remove. This also is said to cause the B vitamins in the outer layers to diffuse into the interior of the grain so that the vitamin content of parboiled rice is not seriously affected by milling.

It is not intended that large numbers of examples of diets should be included in this thesis, but, since reference will be made to the folic acid and vitamin B_{12} content of these diets, further examples are given in Tables 6a & b.

In 1943 there was famine in India, and Patel (1945) carried out a dietary survey in December 1943 in the Bijapur district of Bombay Presidency. His figures, given in Tables 6a & b, are for the dietary intake after the famine was over in a total of 42 families. Those in Group I were the poor labourers in the field and their families. Their annual income of Rs.100 (£7.10.0.) was paid largely in kind. Group II consisted of small agriculturists engaged in casual labour, village artisans and those cultivating land on a rental basis. The annual income was Rs.100 - 300 (£7.10.0. - £22.10.0.).

The main source of protein, even in the non-vegetarians is cereals and pulses.

In their report on anaemia in pregnancy in Calcutta, Napier & Edwards (1941) noted that there was a definite relationship between anaemia and poverty, but were uncertain as to the effects of deficiency of individual constituents of the diet. There was a suggestion that macrocytic anaemia was more common in vegetarians.

The Content of Iron in the Diet.

So far as the iron content of the diet is concerned, we have/

TABLE 6a.

Diet of 42 families investigated by Patel (1945) in Bombay Presidency. (Ounces per person per day).

	Vegetarians		Non-Vegetarians	
	I	II	I	II
Total cereals	19.7	28.8	24.2	26.0
Bajri (cereal)	10.55	16.6	8.05	6.15
Jowar (cereal)	8.94	10.6	11.46	14.5
Rice	0.02	0.18	nil	nil
Wheat	0.36	1.3	nil	nil
Navani (Italian millet)	0.7	nil	nil	nil
Pulses	0.79	1.28	0.59	1.33
Green leaf vegetables	1.5	0.81	2.1	1.36
Ground nuts	2.78	nil	0.26	nil
Milk and milk products	0.3	1.07	0.17	0.85
Butter	0.01	0.06	nil	0.06
Vegetable oils	0.53	0.2	0.2	0.19
Sugar	0.35	0.74	0.24	nil
Condiments (green & red chillies)	1.29	1.3	1.8	2.2
Meat	nil	nil	1.78	1.08
Fish	nil	nil	0.1	0.2
Non leafy vegetables	1.32	1.16	0.72	2.4

TABLE 6b.

Analysis of the Diets shown in Table 6a.

	Vegetarian		Non-Vegetarian	
	I	II	I	II
Calories	2,675	3,177	2,644	2,830
Total protein (g)	80.4	88.6	84.7	89.2
Animal protein (G)	0.3	1.3	10.52	7.2
Carbohydrate (G)	438.5	605.0	504.6	546.9
Fat (G)	34.4	25.1	24.1	25.2
Animal fat (G)	0.9	3.8	3.8	5.3
Calcium (mg.)	0.4	0.5	0.5	0.5
Vitamin A (I.U.)	1,810	1,030	2,520	1,690

have already seen that Macdonald considered that there was a high content of iron in the diet of his coolies. However Goswami & Basu (1938) using the method of Hill as modified by Kohler et al. (1936) for their iron estimations, consider that the diet of the poorer Indian contains only some 5 mg. of iron per day. The daily diet which they analyse is that specified by Aykroyd in Government of India Health Bulletin No.23. It is as shown in Table 7.

TABLE 7.

Available Iron Content of daily diet of Indian Worker (Goswami & Basu, 1938).

		Available Fe (mg.)
Rice (average type eaten)	15 oz.	3.45
Pulse	1 oz.	1.36
Non leafy vegetables	1.5 oz.	0.281
Green leafy vegetables	0.25	0.105
Milk	1 oz.	0.072
		5.27 mg.

This is considered further by Ranganathan (1938a) who believes that the calculation of the iron content of the diet from tables is unsatisfactory. Different workers have greatly different ideas about the iron content of various foodstuffs. In a diet of eight ingredients as used in an Indian home the range of the iron content as estimated by using the published figures of eight workers varied from 10.7 to 51.0 mg. per day. For example, Ranganathan (1938b) considers that parboiled milled rice contains 3.03 mg. per 100 G. of/

of total iron and 0.59 mg. per 100 G. of available iron, whereas Goswami and Basu (1938) give the figures as 0.732 mg. and 0.54 mg. respectively. Both used Kohler's technique for their estimations. Moreover in a huge country like India there is local variation in the iron content of the same foodstuffs and the amount of iron supplied by cooking utensils requires consideration. (In this connection it should be remembered that the poorer Indian prizes brass utensils and eats with his hands).

Indeed the availability of iron from the diet is very complex and little understood. Absorption may be impaired by the high content of phytic acid in the Indian diet. In their 465 cases of anaemia of pregnancy, Napier and Edwards (1941) found that iron therapy was helpful in the least anaemic cases whether the MCV was raised or not, whereas it was of little value in the most anaemic cases whether the MCHC was low or not.

The Content of Folic Acid and Cobalamin Compounds in the Diet.

In many ways knowledge of this may furnish the key to the problem of megaloblastic anaemia in India. Unfortunately, however, we have no information at all from direct assays carried out there. Calculations from tables are most unsatisfactory, as is the case with iron, and for cyanocobalamin we do not even have the tables. There is, however, a detailed account of the folic acid content of various foodstuffs published by the U.S. Dept. of Agriculture (Agricultural Handbook, No.29), and with the kind co-operation of Dr. E.L.R. Stokstad/

Stokstad of Lederle Laboratories, Inc., we have been able to obtain a copy of this book. Measurements quoted in this publication had been made microbiologically.

The main dietary constituent to be considered in the present assessment is rice. It appears that the total folic acid content of 100 G. of uncooked rice (on a fresh weight basis) is of the following order of magnitude:

	L.casei assay	S.faecalis assay
Brown rice	.021 mg.	.024 mg.
Rough rice	.029 mg.	.034 mg.
Milled rice	.014 mg.	.014 mg.

Satisfactory figures for parboiled rice are not available.

If we now refer back to some of the diets already detailed, we find,that, again calculating the total folic acid content of uncooked food from tables, on a fresh weight basis, the following results emerge:

1. The diets of Macdonald's coolie families (Table 4) contain approximately 210 µg. of folic acid if milled rice was eaten, and 330 µg. if 'rough' rice was eaten. (Daily content)
2. The diets of poor families given by Patel (Table 6) contain about 314 µg. and 395 µg. of folic acid respectively for the poorest and better paid vegetarians, but 341 µg. and 368 µg. for the corresponding groups of non-vegetarians. The meat contributed only about 1.1 µg. to 1.6 µg. and the fish 1.0 to 2.0 µg. to the diets of the non-vegetarians. Rice was not the main cereal.
3. The diet of an Indian worker given by Goswami & Basu (Table/

(Table 7) contains approximately 92 µg. of folic acid if milled rice was eaten and 162 µg. if 'rough' rice was eaten.

4. From the same tables, a normal diet of 2,300 calories of the type eaten in the British home contains about 100 µg. of folic acid per day.

When calculated in this way there is little evidence of folic acid deficiency in the Indian diets that have been quoted as compared with the British diet, but unfortunately the matter is by no means as simple as this. In foodstuffs, 'folic acid' may be found as pteroylglutamic acid, citrovorum factor, the conjugate pteroylhexaglutamylglutamic acid, citrovorum factor conjugate and perhaps other unknown forms. In the assays used in the composition of the tables that were employed for the above calculations the foodstuffs had been treated with a chicken pancreas enzyme to release 'free folic acid' from its conjugate form. In fact, however, we do not know to what extent these conjugate forms are available to the body from different foodstuffs; measurements of "free" folic acid by assays in which the foodstuff has not been treated with an enzyme preparation frequently give a lower result than assays that have been performed after enzyme treatment. It is not then certain which is the 'correct' answer.

In the course of the microbiological assay the sample of food is autoclaved at 15 pounds steam pressure for 10 minutes. This in itself does not destroy folic acid, but it may convert

some/

some 'conjugate folic acid' to the 'free' form. More important however, is the fact that it has been shown by Cheldelin et al. (1943) and by Hanning et al. (1944) that there is a very considerable loss of folic acid as a result of cooking. Meat products, other than liver, lose from 46 to 95 per cent of their folic acid content, while vegetable products lose from 69 to 97 per cent with cooking.

As examples of the effects of cooking, according to the data of Cheldelin and his colleagues, there are given in Tables 8 and 9 the folic acid content of the diet of an Indian labourer and a Briton before and after cooking.

It will be seen that the Indian diet has its content of folic acid reduced by 91.4%, whereas the British diet has its content reduced by only 47%. If indeed folic acid deficiency is as common in India as many believe the clue to the dietetic aspect of this may lie in the fact that nearly all the poor Indian's folic acid comes from two sources, cereals and pulses, and that this is always cooked, whereas the European obtains his folic acid from a variety of foodstuffs, some of which are cooked and some raw. On the other hand the fact that the folic acid is no longer available for assay purposes after cooking does not necessarily mean that it has disappeared or even that it is not available to the body, since it may be that it is converted into some bound form. The matter is complicated further by the possibility that certain articles of diet may conceivably contain antimetabolites which render the folic acid non available to the body, either by preventing its/

TABLE 8.

* Total Folic Acid Content of Articles of the Diet of Indian Labourers quoted by Goswami & Basu (1938)

Article of Food	Quantity	Total folic acid content of uncooked food (µg.)	Method of Cooking	Total folic acid content of cooked food (µg.)
Rice, milled	15 oz.	63	steamed 25 mins.	5.04
Pulse (beans)	1 oz.	25	steamed 60 mins.	2.25
Non-leafy vegetables (turnips)	1.5 oz.	2	boiled 40 mins.	0.3
Green leafy vegetables (cabbage)	0.25 oz.	1.4	steamed 30 mins.	0.11
Milk	1 oz.	0.15	-	0.15
TOTAL		91.55		7.85

* The values for uncooked foods have been taken from United States Department of Agriculture Handbook No.29. The degree of destruction of folic acid by cooking has been taken from the report of Cheldelin et al. (1943).

TABLE 9.

* Total Folic Acid Content of Articles of a 2,300 Calorie British Diet.

Article of Food	Quantity	Total folic acid content of uncooked food (µg.)	Method of Cooking	Total folic acid content of cooked food (µg.)
Orange juice	3½ G.	4.8	-	4.8
Cornflakes	2 oz.	2.5	-	2.5
Eggs	2	6.4	scrambled 5 min.	4.2
Milk	20 oz.	4.2	-	4.2
Bread (as such)	6 oz.	25.0	-	25.0
Bacon	2 oz.	5.0	fried 5 mins.	0.4
Meat(round steak)	3½ oz.	6.5	fried 15 mins.	1.4
Potato	6 oz.	16.0	boiled 30 mins.	1.1
Cauliflower	3½ oz.	29.0	steamed 30 mins.	9.0
Apple	4 oz.	0.4	-	0.4
Fish (halibut)	3 oz.	6.4	fried 15 mins.	3.3
Tea, Sugar, Butter		Negl.		Negl.
TOTAL		106.2		56.3

* The values for uncooked foods have been taken from the U.S. Dept. of Agriculture Handbook No.29. The degree of destruction of folic acid by cooking has been taken from the report of Cheldelin et al. (1943).

its absorption or its metabolism in the tissues. No evidence of the existence of such antifolic acid metabolites in the diet has yet been given however.

Since different methods of cooking will vary in their effects on the folic acid in the food, and as we do not know for certain that disappearance of folic acid in the assay as a result of cooking means that it is not available to the body, and since we know nothing about the existence of possible anti-metabolites, or, as will be considered later, the effects of intestinal bacteria on haemopoietic factors, and as we do not know even roughly the minimal daily requirement of folic acid intake by man, it is fair to summarise by saying that we are in no position at present to say to what extent the diet of the poor Indian is deficient in available folic acid. Indirect evidence does suggest, however, that folic acid deficiency exists.

When we come to vitamin B_{12} and related substances, our knowledge is even less. The microbiological assay for cyanocobalamin is a very complex matter, and it is not always certain what is being measured. It does appear, however, that cow's milk contains about 6.6 µg. of cyanocobalamin per litre (Collins et al., 1951) so that the diet given by Goswami & Basu (1938) would have about 0.18 µg. from this source. Cereals contain about 0.2 µg./100 G. (Cuthbertson, W.F.J., 1952), so that in the same diet 0.8 µg. might come from this source. It is not known whether 1 µg. of cyanocobalamin per day is sufficient for man, and we have no knowledge of the effect/

effect of cooking or of the existence in foodstuffs of vitamin B_{12} conjugates. It would, however, appear reasonable to consider it likely that the diet of the poor Indian is deficient in cobalamin compounds.

Conclusions about the Diet.

The diet of the poor Indian is deficient in animal protein and many workers consider that this is one of the main causes of anaemia. In the Belsen concentration camps, however, anaemia was not common. Whether or not it contains a high content of iron is uncertain because of the different views of various workers on the availability of the iron, but certainly there does not seem to be a gross deficiency. Deficiency of folic acid may be due to the fact that the poor Indian derives his folic acid from cereals and pulses and that cooking destroys the folic acid in these. Vitamin B_{12} deficiency also appears to be present. The tissues of the Indian labourer may well be lacking the normal concentration of haemopoietic factors and on this poor soil infection, parasite infestation, pregnancy or even severe exertion itself may cause metabolic changes not found in the well nourished.

NUTRITIONAL MACROCYTIC ANAEMIA

The purpose of this section of the thesis will be to consider the main published papers in the light of modern knowledge and then, after the furnishing of data about our own cases, to give in summary what appear to be the chief aetiological factors in the various parts of the world where nutritional macrocytic anaemia has been investigated.

The/

The most complete accounts of nutritional macrocytic anaemia are those of Wills and of Napier from India, of Fairley, Foy and Kondi from Macedonia, of Trowell from Uganda, and of Foy & Kondi from Kenya.

Other reports that will not be considered in detail are those of Giglioli (1936) from British Guiana, of Groen & Snapper (1937) from Holland, and of Taylor & Manchanda (1940) from India. Many papers have been published from Puerto Rico and the Southern States of the U.S.A. by Spies, Suarez and their co-workers, but since these investigators do not differentiate clearly between nutritional megaloblastic anaemia and tropical sprue, the papers will not be considered in any detail.

NUTRITIONAL MEGALOBLASTIC ANAEMIA IN INDIA.

Early accounts of macrocytic anaemia in India were those of Balfour (1927) who described 150 cases of anaemia in pregnancy and of McSwiney (1927). Thereafter Lucy Wills published her classical series of papers dealing with macrocytic anaemia, particularly in the Bombay area (Wills & Mehta, 1930a, 1930b; Wills & Talpade, 1930; Wills, 1931, 1932; Wills & Billimoria, 1932; Wills, 1933a, 1933b; Wills, Clutterbuck & Evans, 1937; Wills & Evans, 1938).

Many of these reports were headed 'Pernicious Anaemia of Pregnancy" and to a certain extent this has caused readers of the papers to overlook the fact that a similar condition occurs in the non pregnant female and in the male. It is true that many of Wills' patients were pregnant, but the original

aim/

aim of her work was to investigate the importance of anaemia in relation to the high maternal mortality rate in India. Naturally therefore Wills worked in a maternity hospital, but she herself pointed out that the condition was not confined to pregnant women. Indeed in one of her papers (Wills, 1933b) she states that of 61 of her cases of macrocytic anaemia in Bombay, 32 of the patients were males.

Again, in an analysis of various groups of patients (Wills & Talpade, 1930) anaemia was found to be almost equally common in poorer class women who had recently been delivered, non-pregnant patients who had formerly had megaloblastic anaemia of pregnancy, and non pregnant Hindu mill workers. In her earlier papers Wills described the clinical picture and blood findings in her patients and discussed possible causes. The condition was seasonal, occurring chiefly between October and March, but the reason for this was not obvious. The diets were deficient in calories, animal protein, fat and vitamins A, B and C. Balfour (1927), however, had said that the condition was found not only in malnourished Indian women, but also in better-to-do ones. Wills and her co-workers went on to attempt to produce megaloblastic anaemia by dietetic means in the rat (Wills & Mahta, 1930b). The attempts were not successful, but Wills succeeded in producing it in the monkey by feeding a diet of polished rice, white bread and chapatti (Wills & Billimoria, 1932). This led to attempts to cure the anaemia of monkeys. Efforts with vitamins A and C were unsuccessful, but great success attended the therapeutic trial

of/

of the autolysed yeast product 'Marmite'. Wills (1933b) showed that the haemopoietic principle contained in it was water soluble and heat stable in an acid medium. It was not precipitated or inactivated by 80% alcohol. Naturally Wills (1931) then tried Marmite in Indian women with macrocytic anaemia. There was marked improvement, both general and haematological, in the first twenty-two patients treated in this way; eight of them were not pregnant. The dose given was 1 drachm (4 grammes) once, twice, thrice or even four times daily. Liver extracts, at that time relatively crude, were effective by the parenteral route.

The next stage (Wills, Clutterbuck & Evans, 1937) was to treat the monkey anaemia with Anahaemin, a refined liver preparation which had recently been isolated. Parenteral administration of a batch of Anahaemin that had been shown by Ungley to be effective in pernicious anaemia did not cause improvement in the monkey anaemia. It was, however, found that the monkey anaemia responded to the crude liver preparation Campolon. As they had done with the Marmite, Wills and her colleagues (1937, 1938) attempted to find the active fraction of Campolon, and discovered that after treating it with ammonium sulphate they had an insoluble fraction which was active only in pernicious anaemia and a soluble fraction which was effective in pernicious anaemia, monkey megaloblastic anaemia and tropical megaloblastic anaemia. The active principle of Anahaemin was believed to reside also in the/

the ammonium sulphate insoluble fraction of the Campolon. Similarly when autolysed yeast preparations were treated with ammonium sulphate a soluble and an insoluble fraction was obtained. In the monkey anaemia the insoluble factor was inactive, whereas the soluble fraction was effective by mouth. It should, however, be noted that one monkey failed to respond to the soluble extract from Marmite but did respond well to 20 ml. of Anahaemin daily by injection.

The final step in this series of experiments was the carrying out of a trial of Anahaemin and Campolon and other crude extracts in a series of cases of 'tropical macrocytic anaemia'. Both men and women were included in the series. The Anahaemin was first tested on pernicious anaemia patients and found to be potent. In their account of these experiments Wills & Evans give the blood figures, and there is no doubt that the results are clear cut. Anahaemin in doses of up to 2 ml. daily for as long as 12 days by injection was ineffective, whereas Campolon or NeoHepatex, 2 ml. daily was effective in 'tropical macrocytic anaemia'. Two patients did respond to Anahaemin, however.

These therapeutic studies naturally attracted much attention. Mudaliar & Rao (1932), working in Madras reported that their tropical macrocytic anaemia patients responded to liver injections but that there was no response in the twenty cases investigated to Marmite in a dosage of 4 G. t.i.d. Green-Armytage (1932) found that Marmite was effective in nutritional macrocytic anaemia in India, Castle & Rhoads (1932) found it to be effective in some cases of sprue/

sprue, and Vaughan & Hunter (1932) had success in the treatment of macrocytic anaemia in two cases of coeliac disease. In pernicious anaemia there was a divergence of view about the therapeutic value of Marmite. Goodall (1932) was impressed by its value, as was Ungley (1933) but Davidson (1931, 1932) found that only a few cases responded, and Strauss & Castle (1932) found it to be ineffective unless mixed with gastric juice.

So far as liver injections were concerned, Napier et al. (1938) found that Anahaemin injections were effective in some cases of nutritional macrocytic anaemia in Calcutta, while Fraser (1938) stated that in Assam Campolon had been found to be ineffective in macrocytic anaemia of pregnancy if given after the fourth month.

Analysis of these Results.

The main therapeutic problem to be considered in the light of modern knowledge is whether or not 'Wills' factor of Marmite and Campolon is something other than cyanocobalamin or folic acid. So far as Marmite is concerned, the folic acid content is stated by the manufacturers to be 0.004 mg. per gramme, but it must vary from batch to batch. Wills gave some 12 grammes per day which may have been equivalent to about 50 μg. of folic acid. It is not known whether this dose would be sufficient to cause a haematological response in nutritional megaloblastic anaemia — arguments based on the dosage required in pernicious anaemia are invalid since folic acid deficiency is not the primary defect in that condition. If calculations of our daily folic acid intake from the diet

have/

have any meaning, then it would be possible to explain Lucy Wills' results with Marmite on the basis of its folic acid content.

When we come to deal with liver injections, however, the matter is more difficult. The only way to answer the question would be to obtain samples of the batches of the Anahaemin and Campolon used in the experiments, and to measure their content of haemopoietic factor. The author has attempted to obtain such samples, but Wills can no longer provide any, and Messrs. Bayer Ltd. in Britain and in Germany have also been unable to trace samples of the actual batches. Very recently Dr. J.Cox of Messrs. Bayer Ltd. in London has provided the author with ampoules of two batches of Campolon made in Germany just before the 1939-45 War. One batch contained only 0.7 µg. of folic acid substances per ml., but the other contained 4.5 µg./ml. (vitamin B_{12} contents respectively 2.9 and 8.0 µg./ml.)

The first possible explanation of the activity of Campolon and lack of activity of Anahaemin would appear to be that the Campolon had a high content of cyanocobalamin, whereas the Anahaemin was not potent. The objection to this theory is that the Anahaemin had been shown to be effective in pernicious anaemia patients. Moreover, more recent work such as that of Das Gupta et al. (1953) has shown that cyanocobalamin is frequently ineffective in nutritional megaloblastic anaemia. Cyanocobalamin is insoluble at two-thirds saturation with ammonium sulphate (Lester Smith, 1950). Jukes & Stokstad (1948) consider that in her efforts to separate the active fractions of Campolon Wills may well have been separating

cyanocobalamin/

cyanocobalamin from pteroylglutamic acid. We are therefore brought back to the consideration of the folic acid content of liver extracts. In measurements of the folic acid activity of thirty batches of liver extract, including two of Campolon, manufactured in Britain since the 1939-45 War, the author did not find the content of folic acid of any sample to be greater than 0.9 µg. per ml. This is similar to the findings of other workers, and is considerably less than the content of one of the two pre-war batches of Campolon. The content of folic acid in Anahaemin varies from batch to batch, but we have always found it to be less than 0.9 µg./ml. The possible explanations of Wills' results would appear to be:

(a) that the Campolon and NeoHepatex that she used had a much higher content of folic acid than do the modern batches, and possibly a higher content than did the pre-war batches that we have tested.

(b) that pre-war Anahaemin had less folic acid and perhaps even less cyanocobalamin than pre-war Campolon, but that the dosage of folic acid given in the Campolon together with the cyanocobalamin contained in it, was sufficient to give a response in nutritional megaloblastic anaemia.

(c) that another unknown factor (Wills' factor) is present in crude liver extracts.

As yet it is not possible to answer this question. The United States Department of Agriculture Handbook gives the total folic acid content of beef liver (fresh weight) as 294 µg. per 100 G., and in a later chapter it will be seen that this is similar to the author's findings for the folic acid activity of human liver. Thus if all the folic acid were extracted from 15 G. of liver, it would be equivalent to that contained in 12 grammes of Marmite. Whether or not pre-

pre-war Campolon contained anything like 50 µg. of folic acid per ml. cannot now be answered, even by the manufacturers, but it is unlikely. At least it is reasonable to consider that Dr. Wills was injecting 9 µg. daily in her 2 ml. injections, and conceivable that she gave more. We do not know what is the effect of a small dose of folic acid together with a small dose of cyanocobalamin in cases with a deficiency of both.

From Calcutta Napier and his co-workers (1938) described cases of nutritional megaloblastic anaemia in pregnant women and in males. In some patients of both groups there was a response to Anahaemin, but three males failed to respond to it yet showed dramatic improvement with 2 cc. of Campolon daily. It is not, however, clear whether the Anahaemin used was potent, and the information given is insufficient for the possible aetiological factors to be analysed satisfactorily.

In their 1941 account of 'Anaemia in Pregnancy in Calcutta' Napier & Edwards, as we have seen considered poverty to be an important factor. There was, in addition, a marked association between splenomegaly and macrocytic anaemia, and definite correlation between liver enlargement and macrocytosis, and between the serum bilirubin level and macrocytosis. It would appear reasonable to suggest that malaria was a factor here. Napier & Edwards suggested further that the macrocytic anaemia of pregnancy was a form of toxaemia associated with the presence of the foetus, and conditioned by a low dietary intake or deficient absorption of certain essential/

essential blood forming and protecting substances, the syndrome being aggravated by (or perhaps only operative in the presence of) a chronic malarial infection, chronic intestinal infection or diarrhoea. Since a similar condition occurred in males, it seemed possible that the foetus was the "last straw that broke the camel's back".

So far as the influence of pregnancy is concerned, the present author considers, as a result of biochemical observations, that in some instances megaloblastic anaemia of pregnancy may be due to inability to utilise folic acid, presumably as a direct or indirect result of the presence of the foetus. This will be considered in the chapter dealing with the megaloblastic anaemias of pregnancy. (See also p.500)

NUTRITIONAL MEGALOBLASTIC ANAEMIA IN MACEDONIA.

Other accounts of nutritional anaemia that require consideration include those of Fairley et al. (1938) and Foy & Kondi (1939). These workers described nutritional macrocytic anaemia in Macedonia amongst Greek refugees who had fled from Asia Minor some years before. Fairley and his colleagues divided the cases into three sub-groups, non-haemolytic, haemolytic and aplastic. The haemolytic element was attributed to malarial infection. Wills commented in several of her papers that her cases did not have an increase of plasma bilirubin or urinary urobilinogen, but in a discussion after a paper by Hamilton Fairley (1938) she stated that she had excluded such cases from her investigations, regarding them as examples of acquired acholuric jaundice.

In/

In Fairley's series of haemolytic cases the patients all had big spleens. The other important features were that diarrhoea was not a feature, the fat content of the stools was normal, (but the fat intake was low), oral glucose tolerance curves were normal, and the stools did not contain the ova of ankylostome. Fairley and his co-workers considered that poor diet was sufficient to cause the condition in the non-haemolytic cases, but that malaria was a main factor in the haemolytic ones.

The most important known influence that remains to be considered in the causation of nutritional anaemia in these refugees is diet. Detailed diet sheets are not available but the information given about these Macedonian refugees is as follows:

Meat, if taken at all, was taken once a week to once a month. Fish, little or none.

Butter and Eggs - too dear; not taken.

Milk - Given to children and the sick.

Yaghourt (Bulgarian sour milk) - Taken if the refugees could afford it.

Macaroni and Oil of Olives - Taken.

Bread, Olives, Beans were staple diet.

Tomatoes - consumed widely.

Fruit - Large quantities taken, especially grapes, but including wild pears, melons, figs, raisins.

Vegetables - An important element in the diet, both fresh and dried vegetables being taken. Diet included lentils, split peas, onions, garlic, egg plants, pumpkins, cabbages, lettuces, dandelion leaves and many other green-leaf plants.

The diet was stated to be inadequate in calories and

deficient/

deficient in sources of animal protein.

It must be obvious that such a diet has a high content of folic acid and a low content of cobalamin compounds. Accordingly one might predict that this form of megaloblastic anaemia, if largely dietetic in origin, would respond at least as well to Anahaemin as to Campolon. Such indeed, was what happened in megaloblastic anaemia of pregnancy in Macedonia (Foy & Kondi, 1939). A further paper dealing with megaloblastic anaemia in males was promised, but apparently did not appear. In the report of their results of the treatment of pregnancy anaemia, Foy & Kondi include a sentence stating that the condition in males did not respond to crude or refined liver extracts. It may, of course, have been that malaria was a large factor in the production of anaemia in these patients.

In the pregnancy cases, Campolon had to be given in a dosage of 6 - 8 ml. daily and Marmite in a dose of 60 G. daily to give maximal reticulocytosis, and even then the red cell rise was unsatisfactory in the haemolytic type.

Marmite does not contain cobalamin compounds and if indeed the refugees had been taking a diet containing large quantities of folic acid and virtually no cyanocobalamin, it is perhaps not surprising that the folic acid contained in Marmite was ineffective, if indeed folic acid is the important anti-megaloblastic substance in autolysed yeast. (See also p.63).

NUTRITIONAL MACROCYTIC ANAEMIA IN AFRICA.

In 1941 Trowell commenced the writing of a series of papers dealing with nutritional macrocytic anaemia in Uganda. At first he stated that the condition was megaloblastic

(Trowell/

(Trowell, 1942, 1943) but he subsequently stated (1947) that true megaloblasts were not found; this later statement has been confirmed by other workers (Lehmann, 1949). Trowell introduced the term "dimorphic anaemia" to cover a dual deficiency, presumably of antimegaloblastic substances and of iron. The MCHC is reduced in the condition and the MCV is not always raised, but macrocytosis and hypochromia are seen in the peripheral blood film. In Trowell's series of cases described in 1943, there were fifty-three males, two pregnant women and three non-pregnant women. In his later, larger series published at this time and classified according to the MCV (normal 75 - 96 c.μ.) and the MCHC (normal 28 - 34%) the distribution was:

27 cases of macrocytic orthochromic anaemia
63 cases of macrocytic hypochromic anaemia
27 cases of normocytic hypochromic anaemia
11 cases of normocytic orthochromic anaemia
5 cases of microcytic hypochromic anaemia
1 case of microcytic orthochromic anaemia.

In many patients there was splenomegaly and evidence of haemolysis (reticulocytosis and jaundice). Diarrhoea is not mentioned as a feature. The possible aetiological factors that could be discovered were:

Malaria. There was evidence of this in many of the patients and splenomegaly without overt malaria in others.

Ankylostomiasis. Heavy infestation in many of the patients.

Other diseases. Some had taeniasis, ascaridiasis and amoebiasis.

Diet.

Trowell's patients consisted of two groups, the indigenous Ganda, Nyore and Nkole tribes, and the immigrant Ruanda tribes,/

MAP 2. AFRICA.

tribes, members of which had walked from 300 to 600 miles from the Belgian Congo. (See Map 2). The former are peasant agriculturalists who subsist on the staple food of cooked plantains and sweet potatoes, with certain additions of beans and groundnuts. Poorer members of the tribe eat cassava; they have about 3 lbs. of this daily and it is cooked for one to two hours. It supplies about 1,750 calories, 10 G. of protein, no fat and 4 mg. of iron. Before it is cooked, it contains about 450 mg. of ascorbic acid. Nothing is known about its content of folic acid either before or after cooking, and unless it differs greatly from other vegetable substances it is not likely to supply cyanocobalamin. This, however, is a matter that requires investigation since cassava is said to contain cyanide which has to be leached out before it is eaten.

Leafy vegetables are not eaten by these indigenous tribes of Africans and fruit is almost unknown. Moreover meat, fish and milk are almost never taken.

The immigrant Ruandas are in an even more sorry state, earning about fourpence a day if they do obtain work. Deficiency diseases are common.

It will be seen that these men and women are likely to suffer from a combined deficiency of cyanocobalamin and folic acid, in addition to that of animal protein. This conclusion is based on the known fact that the folic acid content of sweet potatoes and beans is practically destroyed by cooking, and the likelihood that this is also true with cassava and plantains.

So far as treatment is concerned, if the condition is largely dietetic and the above conclusions are correct, it would/

would be expected that some patients would respond to pteroylglutamic acid, others to Anahaemin or cyanocobalamin, and the majority more markedly to both. Since pre-war Campolon was no longer available when Trowell did his work, it is not possible to say anything about its effects, and in any event Trowell obtained his refined and crude liver extracts from the United States. It is not at all clear why none of the cases were megaloblastic, but Trowell (1947) is now sure that they were not, and this has been confirmed by Davidson (Personal communication), who saw some of the material. It is a most extraordinary thing that patients with macrocytic anaemia and red cell counts as low as 860,000 per c.mm. (Trowell, 1943) should not show megaloblasts in the marrow. Trowell (1947) appears to be almost in a state of despair about understanding the results of his treatment of these patients. He found good responses to ferrous sulphate therapy in patients with macrocytic orthochromic anaemia and responses to refined liver extract of American manufacture in hypochromic microcytic anaemia. Neither refined or crude extracts were as effective as in pernicious anaemia. Pteroylglutamic acid was not then available, but Trowell (1951) stated that pteroylglutamic acid and cyanocobalamin were not effective in his cases of macrocytic anaemia.

Further papers dealing with anaemia in Uganda have been published by Lehmann (1949, 1952). He considers that the macrocytic cells are reticulocytes and young cells in general, emitted by an efficiently functioning bone marrow into a periphery/

periphery where constant blood loss occurs as a result of hookworm infestation. It is hard to see how this would explain some of the cases described by Trowell (1943b) as having macrocytic anaemia without ankylostomiasis, or macrocytic anaemia without reticulocytosis. Lehmann appears to be attempting to oversimplify a complex problem.

Trowell (1951) described a case of an African with megaloblastic anaemia and considered the diagnosis to be pernicious anaemia; he also reiterated his view that multiple dietary deficiencies were of importance in his cases. For this he was criticised by Hutton (1952) and by Lehmann (1952). In considering these various papers further it would appear reasonable to make the following comments. If the diet is as bad as Trowell suggests, then megaloblastic anaemia should occur, especially where the red cell level is so low. That it does not occur sppears to be a matter upon which experienced haematologists are agreed. Therefore, either the diet is <u>not</u> deficient in folic acid and cyanocobalamin or there is some other source of these available (perhaps from synthesis by intestinal bacteria to an extent not seen in other areas) or the megaloblasts are modified in some way by other influences so that they are no longer recognisable. In the last instance, the megaloblasts must also be prevented by these or other influences from responding to cyanocobalamin or folic acid. If hookworm infestation or malaria is an influence that prevents megaloblastosis then it must operate in Uganda differently to the way it commonly does elsewhere. If there is no deficiency of antimegaloblastic substances, then some other factor must

be/

be operative to an extent not encountered in other areas. It may be a deficiency in the diet or the presence of some inhibitory factor, or malaria or hookworm must be acting in a peculiar way. If none of these explanations hold then either some other known cause of macrocytic anaemia, such as liver disease or chronic infection must operate, or there must be some other factor not commonly encountered elsewhere. It seems unlikely that Lehmann's explanation is the whole story, though no doubt the raised MCV is to a certain extent due to reticulocytosis and the escape of young cells into the peripheral blood. In other areas of the world where ankylostomiasis occurs, hypochromic microcytic anaemia is common. In Uganda it is seldom seen, and it follows that some other influences must be at work.

In recent years Foy & Kondi have published from Kenya a series of papers dealing with nutritional macrocytic anaemia, (Foy & Kondi, 1950, 1952; Foy, Kondi & Hargreaves, 1951a,b,c, 1952; Foy, Kondi, Hargreaves & Lowry, 1952). They have encountered severe anaemia in males, pregnant females and non-pregnant women. The anaemia may be megaloblastic with raised MCV even in the absence of reticulocytosis, megaloblastic with normal MCV and MCHC, or normoblastic with low, normal or high MCV. Some cases had intermediate erythroblasts (Foy & Kondi, 1952). Giant stab cells were said to be characteristic in some of the marrows, but Davidson (1952) points out that these cells are frequently found in iron deficiency anaemia. The patients of Foy et al. also suffered from hookworm infestation and had a poor diet. As will be considered in a later chapter some of them responded to antibiotics, but in addition they had/

had a good remission with oral pteroylglutamic acid or from injections of cyanocobalamin or liver extract, both crude and refined.

These workers do not give sufficient information to enable dietetic calculations to be made but they say that the patients were in the habit of eating a low protein diet with a high carbohydrate content. The patients did not, they say, have clinical evidence of undernutrition, and Foy and his co-workers consider that an abnormal flora of the gut is a main aetiological factor. Such a statement is, however, based only on therapeutic responses to antibiotics. So much stress has been laid on this that it is not possible to analyse other possible factors. Foy and his colleagues do, however, refer to diet, malaria and ankylostomiasis as influences that require consideration. (See also p.253).

RECENT LITERATURE RELATING TO ANAEMIA IN THE EAST*

The most recent accounts include those of Patel (1950) who had some response to cyanocobalamin in nutritional megaloblastic anaemia and in pregnancy megaloblastic anaemia in India (Patel & Kocher, 1950), and of Chaudhuri (1951) who had a response to the same substance in 16 cases of megaloblastic anaemia of pregnancy and the puerperium and four out of five cases of nutritional megaloblastic anaemia in non-pregnant women in New Delhi. Whelan & Cheek (1952) found megaloblastic anaemia in only three out of 2,000 Indians, Malays or Chinese examined in Malaya. Ramalingaswami & Menon (1949) have given an account of the response both to crude liver injections and to pteroylglutamic/

* Published subsequent to our Indian investigations.

pteroylglutamic acid in some cases of nutritional megaloblastic anaemia at Coonoor, South India. Das Gupta et al. (1953) have reported better results with pteroylglutamic acid than with cyanocobalamin in the treatment of nutritional megaloblastic anaemia in Calcutta.

SPRUE.

Spies, Suarez and other workers have written numerous papers in recent years about nutritional megaloblastic anaemia and tropical sprue in the Americas, but as they do not differentiate clearly between the two conditions, an analysis of their cases would not be helpful. Indeed, Moore et al. (1944) in a report on 25 patients with nutritional megaloblastic anaemia seen at Birmingham, Alabama, doubt whether there is any justification for differentiating the condition from sprue. In connection with this there is the finding of Mackie & Fairlie (1929) that in the late stages of tropical sprue the wall of the small intestine becomes atrophic. This is similar to what is found in starvation, the walls of the intestines appearing "like tissue paper" (Sydney Smith, 1949). Thus in the late stages of primary malnutrition there may indeed be little clinical difference from what is found in the late stages of secondary malnutrition from 'sprue'.

ACCOUNTS OF ANAEMIA IN INDIAN ARMY SOLDIERS

It is necessary to mention here the findings of Hynes and his co-workers who were investigating the incidence and type of anaemia amongst soldiers of the Indian army at their depots at the time when on the battlefront, many hundreds of miles away,/

away, megaloblastic anaemia was proving such a problem.

In December 1943 the A.D.M.S. Peshawar District organised a haemoglobin survey of the Indian troops in his area. Of 1,100 men examined only 15 per cent had a haemoglobin level of 14 G. per 100 ml. or more, and 50 per cent had less than 12 G. per 100 ml. A further investigation was ordered, and was carried out by Hynes, Ishaq & Morris (1945a).

These workers found that about a third of the 1,400 men they examined had a haemoglobin level of more than 14 G. per 100 ml., and that 14 per cent had less than 12 G. per 100 ml. The improvement was considered to be due to the addition of meat to the diet. In 341 of the men haemocrit estimations were done, and no case of macrocytic anaemia was found. The anaemia was always hypochromic and usually normocytic.

These men were all natives of North-West India, and included Sikhs and Mohammedans from Patiala; Mohammedans, Jats and Hindus from Rajputana; Mohammedans, Sikhs, Jats and Gujars from the Punjab; and Pathans. They were unselected men and regarded as fit for active service. All had served in the Army for more than one year and two-thirds were in their second or third year of service. The diet was practically vegetarian with a very small daily allowance of meat. The staple articles of food were atta (coarse wheat flour) and dhal (pulses), with some green vegetables.

Hookworm infestation was found in 29 per cent of the 355 men examined for it, but many had iron deficiency anaemia without evidence of ankylostomiasis. The iron deficiency anaemia/

anaemia responded to treatment with ferrous sulphate without deworming having been done. The main feature for consideration appeared to be that the anaemia was present although the men had been eating a diet containing 60 mg. of iron per day in the uncooked form. This iron came almost entirely from vegetable sources and it was assumed that it could not be assimilated.

These men came from a similar population to some of those who were simultaneously developing megaloblastic anaemia on the fighting fronts.

The next investigation by this team was of Indian Army recruits at the I.A.M.C. Training Centre, Rawalpindi, between June and November, 1944. (Hynes, Ishaq & Morris, 1945b). Most of these recruits were ward servants and sweepers, a miscellany drawn from the poorer classes. Full haematocrit determinations were done on the blood of some 600 recruits. In 267 of these men the haemoglobin level was less than 14 G. per 100 ml., but again macrocytic anaemia was almost unknown. The type of anaemia found was normochromic normocytic in 55 per cent of instances, but hypochromia was almost universal in the more severe grades of anaemia. So far as hookworm infestation was concerned, it was concluded that in the presence of other more important causative factors it might increase the degree of anaemia.

The men were given a 4,200 calorie diet containing 57 mg. of total iron, and a supplement of 6 gr. of ferrous sulphate was given daily to half the recruits. In all cases there ensued a tendency towards the development of normal blood figures,/

figures, the low MCHC and MCV rising, and the high MCV falling, with a rise towards higher levels of haemoglobin and red cells where the anaemia was normochromic and normocytic. This improvement occurred more rapidly in the men given the supplement of ferrous sulphate.

In a further experiment carried out in May 1945 on Southern Indian Army recruits at Harihar in Mysore, Hynes, Ishaq, Morris & Verma (1946) examined 801 men. This time the recruits were Tamils and Telegus from Madras Presidency and Malayalis from the south west coast. Rice was the staple article of diet, and very little animal protein had been taken, but the men had been selected by recruiting officers at a time when the supply of recruits exceeded the demand. No longer were men joining the Indian Army to avoid death from starvation, and accordingly these recruits were of a very different calibre to those who would have been seen at Harihar in the previous years. As it was, the mean haemoglobin level was 14.23 G. per 100 ml., and only 45 of the 801 men had levels of less than 13 G. per 100 ml. The anaemia responded to iron even in the presence of hookworm infestation, but malaria or sepsis significantly depressed the response.

A PILOT SURVEY OF ANAEMIA IN INDIAN TROOPS ON ACTIVE SERVICE.

In the conflict against the Japanese between 1942 and 1945, disease caused a tremendous loss of man power to the Allies. The present report is concerned with the Eastern frontoperated by South East Asia and India Commands. No accurate/

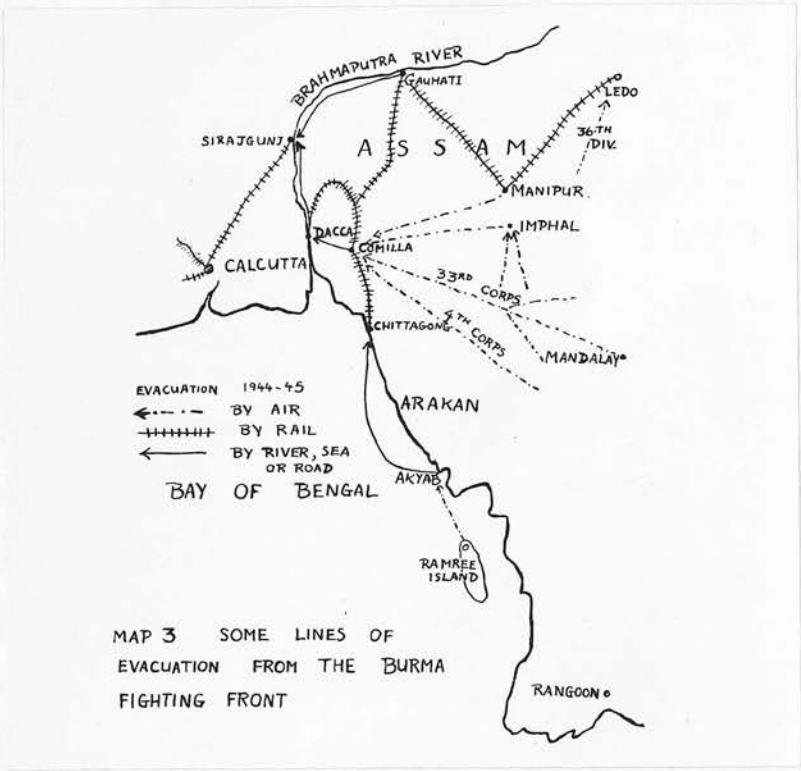
accurate figures are available for illness in 1942, but in 1943 the admission rate to hospitals and other medical units was just under 1,200 per 1,000 per annum, and in 1944 it was about 1,000 per 1,000. The ratio of casualties from sickness to casualties from wounds in 1943 was 121:1 and in 1944, a year of heavy fighting, it was 19:1 (Marriott, 1945). Some units were losing their entire personnel every six to eight weeks from illness.

The greatest medical problem was malaria, accounting for more than half the medical admissions. Diarrhoea and dysentery were common, the former tending to become epidemic in the monsoon seasons. In May, June and July, 1944 the number of cases of diarrhoeal diseases in the 14th Army probably exceeded 100,000.

One problem that gave particular concern because the clinical features were vague, the exact causation uncertain, and the response to all available treatment frequently unsatisfactory, was a sprue-like condition in British and Indian troops with severe anaemia in the Indians. The picture was confused further by the fact that in 1943 the condition coincided with, or was precipitated by, an epidemic of bacillary dysentery. In 1944 this was not so. Where the sprue-like condition predominated, the complaints were of diarrhoea without the occurrence of blood or mucus in the stools, but with abdominal distension, anorexia, sore tongue, weakness and emaciation. The stools were pale and frequently contained an excess of fat. Sometimes there was a response to sulphaguanidine. Anaemia was not found in British personnel to anything/

anything like the extent to which it occurred in Indians, and in the latter very severe macrocytic anaemia was sometimes found without any of the sprue-like symptoms. Sometimes emaciation occurred without anaemia and sometimes anaemia without emaciation. The present author's first introduction to the problem was in June 1944 when he was shown two wards in a Base Hospital at Delhi filled with Indians evacuated from Assam or Burma on account of anaemia or the "marasmus syndrome". There had been five deaths in the preceding fortnight despite all efforts at treatment with diet, autolysed yeast, liver injections, blood transfusions, vitamins and anti-malarial drugs. At post mortem in the emaciated cases there had been found generalised atrophy of all organs including the walls of the intestine.

The object of the survey now to be described was simply to define the type or types of anaemia present and the clinical pictures. The investigation was to be confined to Indian troops. Therapeutic trials were to be considered later, and plans for this further research work were made, but, as has already been mentioned, the collapse of the war put an end to organised research and the clinical picture gradually disappeared when the fighting ceased. For this reason the therapeutic experiments described here were of a very preliminary nature. Most of the cases were seen at a transit hospital at Sirajgunj in Bengal between December 1944 and February 1945. The patients were usually accommodated here for only a few days or even hours in the course of their evacuation from the fighting front to the base hospitals. This site was chosen for/



MAP 3 SOME LINES OF EVACUATION FROM THE BURMA FIGHTING FRONT

The Main Street, Sirajgunj.

for the investigation because several lines of evacuation converged upon Sirajgunj. The chief objection from the research point of view was the shortness of stay of patients in the hospital, but in any event, at this stage the object was to see as many cases as possible. Other cases included in Table 14 were seen at a hospital in Dacca, Eastern Bengal, again in the course of their evacuation to the Base. The wards in both hospitals were straw roofed bashas, and the equipment consisted of a high speed electric centrifuge obtained from the United States, a Sahli haemoglobinometer standardised by Dr. Martin Hynes against an accurately standardised Zeiss haemometer so that 100% was equal to 14.8 G. per 100 ml., and standardised American red cell and white cell pipettes. No biochemical laboratory service or equipment was provided; assistance was available from a R.A.M.C. sergeant, but, unfortunately, he himself spent much of the time in hospital with malaria or dysentery. For these reasons the scope of the investigation had to be limited, but it is felt that the information collected should be presented here, in an abbreviated form, as the problems of the megaloblastic anaemias in Assam and Burma differed in some ways from those in the cases described by workers in other areas. It was not, of course, possible at the time to consider these cases in terms of folic acid or vitamin B_{12} metabolism as pteroylglutamic acid had not been synthesised and vitamin B_{12} was unknown.

It should be stressed that patients were not evacuated from/

from the fighting front simply because they had uncomplicated malaria or dysentery. Evacuation of medical or surgical cases to Base Hospitals was carried out only where absence from the man's unit was likely to be prolonged because of the severity of his illness.

As a background to the main investigation, a haemoglobin survey was carried out in May 1945 on 500 consecutive admissions to the hospital at Sirajgunj. This survey was confined to Indian other ranks, both surgical and medical cases being included. The mean haemoglobin level was 14.17 G. per 100 ml., and the distribution was as in Table 10.

TABLE 10.

The Distribution of Hb. Values (Capillary Blood) in a Hospital Population of Indian Troops.

Haemoglobin (grammes per 100 ml.).

	3-	4-	5-	6-	7-	8-	9-	10-	11-	12-	13-	14-	15-	16-	17-	Total	Mean
No. of Cases	1	0	1	3	7	11	12	20	21	34	58	105	150	56	21	500	14.17
Per cent	←		2.4	→		2.2	2.4	4	4.2	6.8	11.6	21	30	11.2	4.2		

The figures are analysed further in the following tables. There is no evidence that dietetic habits affected the haemoglobin levels at this stage of the campaign.

It will be seen that the men of the Pioneer Corps were significantly more anaemic than the others. These Pioneers came from a recently recruited unit.

TABLE 11./

TABLE 11.

Influence of Diet on the Haemoglobin Level of 500 Indian Other Ranks.

Group	Mean Hb. (G. per 100 ml.)	No. of Cases	Standard Deviation
Rice eaters (A)	13.966	58	2.6446
Atta eaters (B)	14.347	121	2.3388
Atta & rice both eaten (C)	14.157	321	2.3260
Non meat eaters (D)	14.022	60	2.7258
Meat eaters (E)	14.202	440	2.3389

Analysis of these figures. (using the 't' test - Fisher 1944).

Groups compared	t	P	Significance
A B	0.94	0.3	Not significant
A C	0.76	0.45	Not significant
B C	0.53	0.6	Not significant
D E	0.49	0.6	Not significant

TABLE 12.

Haemoglobin Levels according to branch of Service.

Branch		Mean Hb. (G. per 100 ml.)	No. of Cases	Standard Deviation
Infantry	(F)	14.276	131	2.2036
Pioneers	(G)	13.252	107	2.7621
Service Corps	(H)	14.598	52	2.5257
Other Ancillary Units	(I)	14.487	210	2.0726

Analysis of these figures.

Groups compared	t	P	Significance
F G	3.1	< 0.01	Highly significant
F H	0.81	0.4	Not significant
F I	0.88	0.4	Not significant
G H	3.1	< 0.01	Highly significant
G I	4.1	< 0.01	Highly significant
H I	0.29	0.8	Not significant

TABLE 13.

Haemoglobin Levels according to Caste or Religion

		Mean Hb.(G.per 100 ml.)	No. of Cases	Standard Deviation
Brahmins	(J)	14.407	27	2.6092
Jats	(K)	13.600	34	3.3000
Rajputs	(L)	14.546	35	1.5107
Sikhs	(M)	14.343	28	1.9314
Madrasis	(N)	14.685	61	1.7113
Mahommedans	(O)	14.262	156	2.4100
Gurkhas	(P)	14.316	32	1.7294
Christians	(Q)	14.250	12	2.3513
Other Hindus	(R)	13.724	115	2.5560

Analysis of these figures.

Caste		J	K	L	M	N	O	P	Q	R
(J)	t	-	1.7	0.25	0.10	0.51	0.27	0.15	0.19	1.2
	P	-	0.1	0.8	0.9	0.6	0.8	0.9	0.9	0.2
	*S	-	NS	NS	NS	NS	NS	NS	NS	NS
(K)	t	-	-	1.6	1.1	1.8	1.1	1.1	0.74	0.20
	P	-	-	0.1	0.2	0.07	0.2	0.2	0.5	0.8
	S	-	-	NS	NS	NS	NS	NS	NS	NS
(L)	t	-	-	-	0.45	0.12	0.90	0.58	0.41	2.7
	P	-	-	-	0.6	0.9	0.3	0.6	0.7	0.01
	S	-	-	-	NS	NS	NS	NS	NS	DS
(M)	t	-	-	-	-	0.80	0.20	0.06	0.12	0.14
	P	-	-	-	-	0.4	0.8	0.9	0.9	0.9
	S	-	-	-	-	NS	NS	NS	NS	NS
(N)	t	-	-	-	-	-	1.4	0.98	0.61	3.1
	P	-	-	-	-	-	0.1	0.3	0.5	< 0.01
	S	-	-	-	-	-	NS	NS	NS	HS
(O)	t	-	-	-	-	-	-	0.15	0.02	0.18
	P	-	-	-	-	-	-	0.9	0.9	0.9
	S	-	-	-	-	-	-	NS	NS	NS
(P)	t	-	-	-	-	-	-	-	0.09	1.5
	P	-	-	-	-	-	-	-	0.9	0.1
	S	-	-	-	-	-	-	-	NS	NS
(R)	t	-	-	-	-	-	-	-	-	0.73
	P	-	-	-	-	-	-	-	-	0.5
	S	-	-	-	-	-	-	-	-	NS
(R)	t	-	-	-	-	-	-	-	-	-

* S = Significance
NS = Not significant
DS = Doubtful significance
HS = Highly significant

TABLE 14a. ANAEMIA IN INDIAN SOLDIERS ON ACTIVE SERVICE.

TABLE 14a. ANAEMIA IN INDIAN SOLDIERS ON ACTIVE SERVICE.

CASE	DIARRHOEA	GLOSSITIS (G). ATROPHIC (A). PAINFUL TONGUE (P)	MALARIAL PARASITES ISOLATED. (L = AFTER LIVER INJECTION)	FEVER, IF NO MALARIA	PREVIOUS MALARIAL HISTORY IF NO MALARIA	WEIGHT LOSS	LOWEST WEIGHT RECORDED	SPLEEN PALPABLE (T = TENDER)	ANKYLOSTOME OVA ISOLATED	UNIT FOOD *	NO. OF TIMES PER WEEK MEAT SUPPLIED AT UNIT *	MEAT EATER	LENGTH OF SERVICE (YEARS)	SERVICE IN EASTERN REGIONS (MONTHS) *	PREVIOUS TREATMENT AND RESPONSE AMT = ANTI MALARIAL TREATMENT AY = AUTOLYSED YEAST O.L. = ORAL LIVER EXTRACT HEP = HEPASTAB TCF = TCF LIVER EXTRACT LIVER = LIVER EXTRACT (Unspecified)	SERUM ICTERIC	HB. (G.%)	RBC (Mills./c.mm.)	MCV (c.)	MCHC (%)	WBC (per c.mm.)	RETICULOCYTES (%)	MARROW: M = MEGALOBLASTIC. N = NORMOBLASTIC	INTERMEDIATE MEGALOBLASTS AS %AGE OF TOTAL RED CELL PRECURSORS	LEUCO/ERYTHROBLASTIC RATIO
1	+	-	MT			++		+	-	G	V	Yes	2½	12 Assam	Prev. Nil to AMT. Now Resp. to Blood and TCF	+	2.8	0.395	177.0	35.0	1,040	3.75	M	11.5	0.5:1
2	+	P:A	BT:MT.(L)			+	98	-	-	G		No	3	26 Assam	Prev. Nil to TCF (12 ml.), AY. Now slow to HEP + AMT 1	-	2.9	0.835	131.7	26.4	3,100	< 1	M	21.3	1:1
3	+	-	-	+	-	±	-	+	-	G	V	No	2¼	10 Assam	Prev. Nil to 24 ml. liver inj. and AMT	-	3.7	0.864	138.9	30.8	1,200	< 1	M	1.1	1.2:1
4	-	-	-	-	-	±		-	-	G	Occas.	Yes	4	4 Assam 5 Burma	Good to TCF + AMT 1 together	-	5.1	1.023	142.7	34.9	4,000	2.75	M	35.1	0.5:1
5	+	-	BT			±	107	T	+	G	?	Yes	2	5 Arak	Prev. Nil to AY, fresh liver, AMT. Now Nil to AMT 1, HEP.	-	2.9	1.09	83.4	31.9	3,000	< 1	M	2.9	2.0:1
6	-	-	-	-	-	-	131	-	-	G		No	3½	36 Chit.	-	-	5.3	1.19	137.8	39.3	2,100	< 1	M	7.8	0.3:1
7	-	-	BT			-	132	++	-	G		Yes	2¾	4 Burma	-	-	5.9	1.26	127.0	36.9	2,400	1	M	1.6	0.7:1
8	+	+	BT			±		++	-	G		No	2	9 Assam	Prev. Nil to AMT, liver inj. Now good to Blood, HEP, AMT 2	-	6.1	1.28	100.0	33.7	3,800	< 1	M	9.5	0.6:1
9	+	G	-	-	-	++	80	-	-	G		No	4	12 Assam	Prev. Nil to O.L. & AY for 2 mths.	-	5.2	1.29	134.1	30.0	5,400	< 1	M	1.7	0.6:1
10	-	A	MT			±		-	-	G	V	Yes	3	2 Assam	Prev. Nil to 14 ml. liver; AY. Now poor to HEP, AMT 2	+	5.8	1.32	120.2	36.0	5,400	6.3	M	3.0	0.4:1
11	-	P	BT.(L)			-		+++	+	G		No	2¾	8 Chit.	Good to HEP & AMT 2	+	6.3	1.32	159.1	30.0	4,900	2.8	M	41.0	0.3:1
12	-	A	BT.(L)			-	135	-	-	G		Yes	4	6 days Chit.	Nil to HEP, AMT 1	-	5.8	1.33	127.8	34.1	3,800	1.25	M	21.1	0.6:1
13	+	-	BT.(L)			±	103	-	-	G		Yes	3	3 Chit. 12 Burma	Nil to HEP, AMT	-	5.1	1.41	115.6	31.3	2,100	2.0	M	8.0	0.4:1
14	-	-	-	+	+	+		-	+	G	V	Yes	3	6 Assam	Fair to TCF	+	5.8	1.41	130.9	31.4	8,200	1.75	M	6.7	2.0:1
15	+	-	BT.(L)			-		+	-	G		Yes	7	12 Burma	Prev. Nil to AY, 28 ml. liver inj. Now Good to HEP, AMT 2	-	6.8	1.43	137.2	34.5	3,500	2.75	M	17.9	1.2:1
16	-	-	MT			-	96	+	-	G		No	1½	4 Chit.	Prev. Nil to AMT. Now fair to HEP.	-	4.6	1.44	-	-	2,400	6.5	M	19.2	1.1:1
17	-	-	-	-	+	-	104	+	-	G		No	3½	36 Assam	-	-	5.2	1.46	103.8	34.2	1,600	< 1	M	12.2	1:1
18	-	A	-	-	+	+		-	-	G	2	Yes	3	30 Burma	Fair to HEP	+	5.6	1.48	123.6	30.6	2,800	< 1	M	14.0	0.3:1
19	+	A	BT			+		-	-	G	1	Yes	2	12 Assam	Prev. Nil to AY, O.L., AMT.	-	6.2	1.50	126.9	32.6	3,500	< 1	M	8.3	1.4:1
20	+	G	BT.(L)			+		T	-	G	1	Yes	10	12 Assam	Prev. Nil to AY. Now good to HEP, AMT 2	-	6.8	1.59	135.2	31.6	1,500	< 1	M	43.3	1.3:1
21	+	P	BT:MT.(L)			+		-	+	G		No	4	24 Assam	Poor to AMT 1, HEP, AMT 2	-	6.9	1.68	121.8	33.8	4,700	< 1	M	7.6	1.3:1
22	+	A	-	+	+	+	108	T	-	G		Yes	3	24 Assam	Prev. Nil to AMT + Sulphaguan.	-	8.2	1.74	138.3	34.2	4,300	1	M	2.4	3:1
23	+	-	-	+	-	++		+	-	G	7	Yes	3	4 Assam	Prev. Nil to Liver inj. 32 ml.	-	5.8	1.79	121.8	26.7	2,300	< 1	M	1.0	0.6:1
24	+	-	BT			+	109	-	-	P		Yes	4	2 Burma	Nil to HEP, AMT 1	-	7.2	1.80	124.4	32.6	2,100	3.2	M	18.3	1.3:1
25	-	P	-	+	+	-		-	-	G		No	2½	24 Assam	Prev. Nil to AY, Liver inj. fresh liver. Now Fair to HEP, AMT 1	-	6.1	1.81	100.0	33.7	3,800	< 1	M	22.6	1.6:1
26	+	P:A	-	-	+	+	98	-	-	P	1	Yes	3	12 Assam	Prev. Nil to O.L. & AY	-	8.0	1.88	132.9	32.0	3,000	1.6	M	0.5	0.9:1
27	-	G	-	-	+	+		-	-	G	1	Yes	1½	18 Assam	Prev. Nil to AY, Liver inj. Now Slow to TCF, AMT 1, HEP	-	9.4	1.99	159.5	30.0	5,000	< 1	M	11.5	2.8:1
28	+	G	BT			-		-	-	G	7	Yes	2	18 Burma	-	-	8.8	2.00	128.7	34.1	3,000	< 1	N		2.2:1
29	+	A	BT					+	-	VG	7	Yes	2½	18 Assam	Prev. Nil to 38 ml. TCF, O.L., AMT	-	7.4	2.01	137.3	26.9	4,800	1	M	12.3	2.7:1
30	+	-	MT.(L)			+		T	+	G		Yes	2	18 Burma	Prev. Nil to Liver inj.(20 ml.) AMT, deworming; now Nil to HEP	-	4.4	2.07	70.0	30.3	13,600	1.75	N		2.0:1
31	+	-	BT.(L)			+	82	T	-	G		Yes	3	3 Burma	Good to HEP, AMT 1	-	8.6	2.08	-	-	4,800	8.25	N		3.1:1
32	+	G	-	-	+	++		-	-	G	2	Yes	1½	16 Chit.	Slow to TCF, HEP	-	7.9	2.10	121.7	33.0	3,400	< 1	M	14.8	2.1:1
33	+	A	BT			+	85	-	-	G		No	5	4 Burma	Nil to AMT 1	+	8.1	2.21	105.4	34.8	2,300	< 1	M	10.9	1.1:1
34	-	-	MT			±	100	+	-	G		Yes	2	4 Chit.	Slow to AMT 1 & HEP	-	6.7	2.25	92.6	32.2	2,700	2.0	N		1.6:1
35	-	-	BT			-		++	+	VG		-	4	6 Assam	Prev. Fair to AMT, Transfusion	-	8.0	2.25	116.7	30.5	9,100	6.75	N		1.0:1
36	-	-	MT			-	-	++	+	G		Yes	2	18 Assam	-	+	8.8	2.25	120.0	32.6	4,900	6.75	M	1.5	2:1
37	-	G	-	+	+	+	106	-	+	G		No	4	6 Burma 26 Assam	-	-	10.5	2.35	120.0	34.3	4,000	< 1	M	3.4	3.6:1
38	-	-	-	+	-	±		-	+	P	0	Yes	4	12 Burma	Prev. Nil to AT, O.L. Now Fair to TCF	-	10.4	2.36	131.4	33.6	5,000	< 1	N		1.7:1
39	-	A	-	-	-	-	92	-	+	G		Yes	3	10 Assam	Prev. Nil to O.L. Now Nil to HEP	-	10.0	2.37	119.0	35.5	3,000	< 1	M	7.0	1.6:1
40	-	P	-	-	-	+	108	-	-	G		Yes	2¼	24 Chit.	-	-	8.4	2.38	104.6	33.7	3,200	< 1	M	2.0	0.6:1
41	-	-	BT.(L)			±	98	-	+	P		Yes	1½	1 Chit.	Nil to HEP, AMT 1	+	4.2	2.40	75.0	23.4	2,300	4.0	N		0.7:1
42	-	-	BT			+		-	-	VG	7	Yes	2½	24 Assam	Prev. Nil to AY, oral liver, AMT, 12 ml. liver inj.	-	11.0	2.43	135.3	33.5	3,200	< 1	M	5.5	3.3:1
43	-	-	-	-	-	-	134	+	-	G		Yes	2	4 Chit.	-	-	8.5	2.53	122.5	27.4	3,800	< 1	M	1.7	0.2:1
44	-	-	BT			+		-	-	G		Yes	7	6 Assam & Burma	Slow to extensive AMT	+	10.0	2.55	105.9	36.3	3,800	< 1	N		1.5:1
45	+	-	BT	-	-	±	95	-	-	G	7	Yes	3	8 Assam 9 Arak	Prev. Nil to AMT	-	11.6	2.64	132.6	33.0	4,600	< 1	N		1.9:1
46	+	-	MT			-		-		P	0	Yes	3½	12 Assam	-	-	9.1	2.66	109.0	31.4	5,900	1	M	1.0	1.1:1
47	+	A	-	-	+	±		-	+	G	7	Yes	3	24 Assam	Prev. Nil to 24 ml. Liver inj.	-	12.4	2.66	139.0	33.5	5,100	< 1	N		2.5:1
48	+	G	-	-	-	+		-	-	P		Yes	3	6 Burma	Nil to HEP, AMT 1	-	10.4	2.69	133.3	29.0	7,900	3.5	N		1.6:1
49	-	-	MT & BT			-		+++	++	G	7	Yes	2	6 Assam	Prev. Good to AMT		6.9	2.73	88.2	28.6	2,900	< 1	N		1.3:1

* P = Poor; G = Good; V = Variable; Chit. = Chittagong; Arak = Arakan

TABLE 14b. ANAEMIA IN INDIAN SOLDIERS ON ACTIVE SERVICE.

TABLE 14b. ANAEMIA IN INDIAN SOLDIERS ON ACTIVE SERVICE.

CASE	DIARRHOEA	GLOSSITIS (G). ATROPHIC (A). PAINFUL TONGUE (P)	MALARIAL PARASITES ISOLATED. (L = AFTER LIVER INJECTION)	FEVER, IF NO MALARIA	PREVIOUS MALARIAL HISTORY IF NO MALARIA	WEIGHT LOSS	LOWEST WEIGHT RECORDED	SPLEEN PALPABLE (T = TENDER)	ANKYLOSTOME OVA ISOLATED	UNIT FOOD *	NO. OF TIMES PER WEEK MEAT SUPPLIED AT UNIT *	MEAT EATER	LENGTH OF SERVICE (YEARS)	SERVICE IN EASTERN REGIONS (MONTHS) *	PREVIOUS TREATMENT AND RESPONSE AMT = ANTI MALARIAL TREATMENT AY = AUTOLYSED YEAST O.L. = ORAL LIVER EXTRACT HEP = HEPASTAB TCF = TCF LIVER EXTRACT LIVER = LIVER EXTRACT (Unspecified)	SERUM ICTERIC	HB. (G.%)	RBC (Mills./c.mm.)	MCV (c.l.)	MCHC (%)	WBC (per c.mm.)	RETICULOCYTES (%)	MARROW: M = MEGALOBLASTIC. N = NORMOBLASTIC	INTERMEDIATE MEGALOBLASTS AS %AGE OF TOTAL RED CELL PRECURSORS	LEUCO/ERYTHROBLASTIC RATIO
50	+	+	MT		±		-	+	P	V	Yes	3½	10 Burma	Slow to AMT 2	+	9.8	2.75	92.8	38.4	3,800	< 1	M	1.4	1.8:1
51	-	-	-	+	- ±		-	-	G		No	2¾	4 Assam	Prev. Slight with O.L. 12 ml. Liver inj.	-	10.0	2.82	124.8	28.4	3,600	1	N		2.1:1
52	-	G	-	-	+ ±	100	-	+	G	2	Yes	3	10 Assam	-	-	13.2	2.83	143.4	33.0	4,800	< 1	M	0.6	1.4:1
53	-	P	-	-	- ±	103	-	-	G		No	3	5 Assam	Prev. Nil to 16 ml. Liver inj.	-	11.0	2.83	121.9	31.9	6,800	< 1	M	7.0	2.1:1
54	-	-	-	-	- ++		-	-	G	V	Yes	1	12 Assam	-	-	11.8	2.98	122.5	29.9	4,000	< 1	N		4.6:1
55	-	-	-	+	- -		-	-	G		No	2	9 Assam	Slow to HEP	-	12.2	2.99	123.7	33.0	11,200	< 1	N		1.7:1
56	+	G	-	-	+ +	91	-	-	G	1	Yes	8	18 Assam	Prev. Nil to AY	-	12.2	3.0	125.0	32.1	5,700	< 1	M	13.75	4.0:1
57	+	G	-	+	+ -		T	-	G		Yes	2½	2 Assam 12 Chit.	Prev. Nil to AY	-	11.1	3.0	114.1	32.4	4,500	5.0	M	2.8	0.6:1
58	-	-	-	+	- -		-	+	P		Yes	4	36 Assam & Burma	Prev. Nil to O.L.	-	10.0	3.07	107.5	30.3	2,800	< 1	N		2.2:1
59	+	G	-	-	+ ++	79	T	-	G	1	Yes	10	3 Assam	Prev. Fair to 42 ml. liver ext.	-	10.9	3.08	123.3	28.7	6,300	< 1	N		1.9:1
60	+	G	-	-	+ +		-	-	G	V	Yes	2¾	12 Assam	-	-	9.2	3.09	113.3	26.3	4,700	< 1	N		1.4:1
61	-	-	MT		±		-	+	G	0	Yes	2	12 Burma	Prev. Nil to AMT	-	8.4	3.11	106.0	25.5	10,600	< 1	N		1.1:1
62	+	P	BT	-	- ±		+	-	P	V	Yes	4	35 Assam	Prev. Good to AMT, O.L., Blood		10.4	3.15	116.7	30.0	5,100	3	N		0.7:1
63	+	P	-	+	+ ±	84	T	-	G		Yes	4	9 Assam	-	-	12.4	3.16	116.9	33.6	3,500	1.0	N		2:1
64	-	-	BT.(L)		+	89	-	+	P		No	½	6 Chit.	Poor to HEP, AMT 1	-	8.6	3.20	90.0	29.9	5,400	1.5	N		1.7:1
65	+	-	MT.(L)		+	95	-	-	O	O	Yes	2	12 Assam	Prev. Nil to AMT, AY. Now Poor to HEP, AMT 2	-	11.0	3.25	110.8	30.6	8,100	< 1	M	3.0	1.4:1
66	-	-	BT.MT		+		++	-	G	7	Yes	2½	3 Assam 3 Arak 2 Burma	Prev. Fair to AMT	-	9.8	3.28	91.5	32.7	10,700	3.3	N		2.8:1
67	-	-	BT		-	120	+	+	G		Yes	2½	3 Arak 8 Burma	Nil to HEP	-	8.8	3.28	82.9	32.5	5,600	2	N		6.5:1
68	+	-	-	+	+ ±		-	-	P	O	Yes	3	24 Burma	-	-	10.4	3.31	100.6	30.1	4,500	< 1	N		6.5:1
69	-	-		-	+ -		T	-	G	O	Yes	2¾	24 Burma	-	-	12.4	3.37	112.7	32.6	9,300	< 1	N		1:1
70	+	-	-	-	- -	100	-	-	G	3	Yes	2½	12 Assam	-	-	12.0	3.47	112.6	30.8	4,900	< 1	N		1.8:1
71	+	-	BT		-		-	+	G		Yes	¼	1 Assam	-	-	9.2	3.47	89.5	29.7	10,400	4.25	N		2.9:1
72	+	G	-	+	+ +	80	-	-	G		No	2¾	30 Assam	Prev. Fair to AMT 1, Protein Hydrolysate	-	10.6	3.47	115.1	26.5	12,000	< 1	N		4.9:1
73	+	-	-	-	- ±		-	-	G	V	Yes	3	24 Assam	-	-	13.6	3.51	119.7	32.4	6,300	< 1	M	14.3	3.2:1
74	-	-	-	+	+ +		++	+	G		No	3	32 Assam	Prev. Good to AMT, Blood		13.6	3.53	130.2	30.2	3,200	3	N		1.3:1
75	-	-	-	-	- -		++	-	G		No	2	Nil	-	-	12.0	3.53	114.7	30.0	5,300	< 1	N		2.3:1
76	+	G	-	-	- -	120	-	-	G	1	Yes	3	13 Assam	-	-	12.6	3.56	115.2	30.9	2,200	2.5	N		1.1:1
77	+	G	-	-	- +	104	-	-	VG	3	Yes	3	17 Assam	Prev. Nil to AMT		13.0	3.58	111.9	32.5	6,300	< 1	N		1.8:1
78	-	-	BT.(L)		-	121	-	-	G		Yes	1	3 Chit.	Fair to HEP, AMT 1	-	6.5	3.61	69.3	26.0	3,100	2.5	N		1.1:1
79	+	-	BT.(L)		+	95	-	-	G		Yes	2	12 Chit.	Nil to HEP, AMT 1	-	8.8	3.62	80.1	30.3	4,500	< 1	N		4.8:1
80	-	-	-	-	+ -			+	G		Yes	¼	3 Assam	Prev. Good to AY, O.L., deworming. Now Good to HEP, AMT	-	11.4	3.62	120.2	26.2	9,400	< 1	N		1.6:1
81	-	-	-	+	+ -			+	G	2	Yes	2	20 Assam	-	-	10.6	3.65	94.7	27.5	3,100	< 1	N		2.3:1
82	+	-	-	-	- -		-	-	P		No	3½	36 Assam	Prev. Good to O.L., Blood, Liver ext.	-	13.0	3.66	120.1	29.5	4,800	< 1	N		3.0:1
83	-	P	BT	-	- +		++	-	G	1	Yes	2	3 Burma	-	-	10.4	3.80	94.0	29.0	4,900	4	N		1.2:1
84	-	P	-	+	+ +		T	-	G		No	3½	11 Arak	-	-	11.0	3.89	93.6	30.0	2,800	< 1	N		1.4:1
85	+	-	-	+	- ++		-	+	G		Yes	¼	?	-	-	5.6	3.97	68.0	16.3	5,700	2.5	N		1.2:1
86	-	-	-	-	- -	110	-	+	G	2	Yes	3	6 Assam 12 Arak	-	-	7.4	3.99	71.4	25.9	5,900	5.5	N		0.8:1
87	-	-	-	+	- -		-	-	VG	7	Yes	3	30 Assam	Prev. Good to 12 ml. liver inj.	-	8.4	4.02	72.1	29.0	4,200	5	N		1:1
88	+	-	-	+	+ -		++	-	G	1	Yes	2	20 Assam	-		10.2	4.04	93.6	27.0	8,600	< 1	N		1.4:1
89	-	-	-	-	+ -		-	-	G	7	Yes	2	2 Assam	Prev. Good to AMT	-	8.0	4.09	82.9	23.6	4,300	< 1	N		2.3:1
90	-	-	BT		-		-	+	G	5	Yes	2	7 Assam	Prev. Good to AMT, O.L., AY	-	7.9	4.11	80.4	23.2	8,100	< 1	N		2.9:1
91	-	-	-	-	- -		++	+	G	7	Yes	¼	1 Assam	-	-	6.4	4,20	73.8	20.7	4,300	< 1	N		2.1:1
92	+	G	-	-	- ±		-	-	G	2	Yes	3¾	15 Assam & Burma	Prev. Good to AMT, AY	+	13.4	4.20	100.0	32.0	6,000	< 1	N		2.6:1
93	-	G	-	-	+ ±		-	-	G		No	4	4 Burma	-	-	13.2	4.30	102.1	30.0	7,300	1	N		1.3:1
94	+	-	-	-	+ -		+	-	G		No	13	8 Burma	-		13.2	4.52	159.5	30.0	5,100	< 1	N		0.9:1
95	-	-	BT		-		++	-	G	7	Yes	1	Nil	-	-	13.1	4.66	88.0	31.9	8,100	< 1	N		1.5:1
96	-	P	-	+	- ±		++	-	G	1	Yes	3	3 Burma	-	-	11.4	4.68	90.2	27.0	7,900	3.75	N		5.3:1
97	-	-	-	-	+ -		-	+	G	V	Yes		1 Chit.	-	-	10.0	4.72	80.4	26.3	6,500	< 1	N		2.1:1

* P = Poor; G = Good; V = Variable; Chit. = Chittagong; Arak = Arakan

The only difference seen here is that the 'Other Hindus', which includes most of the Pioneers mentioned above, are more anaemic than the Madrasi Hindus and possibly the Rajputs.

When the figures were analysed according to years of service, the men with less than a year's service were significantly more anaemic than all the other groups, their mean haemoglobin being 11.89 G. per 100 ml. This was again due to the inclusion of the Pioneers. Hookworm infestation was a probable important reason for the anaemia in the latter.

Only 14 of the 500 men were being evacuated with a primary diagnosis of malaria, but a malarial history was obtained in 273 of them and many more had no doubt been infected. It should be stressed that all these men had been under orders to take suppressive mepacrine daily in 1944 and 1945 if in the Eastern regions or on leave from them.

MAIN PILOT INVESTIGATION.

In Table 14 there is given a summary of the main particulars of 97 anaemic patients whose investigation included sternal puncture with differential marrow count. These men had all been serving in areas east of the Brahmaputra river, and were being evacuated to base hospitals with diagnoses such as "nutritional anaemia", "malarial saturation", "malnutrition", "sprue", "Parasprue", or "avitaminosis". In general the clinical features included weakness, anorexia, breathlessness, diarrhoea, painful tongue and abdominal discomfort. The Indian soldier is very vague about symptoms and about time so that symptoms have been largely omitted from Table 14, and the duration of the illness has not been included.

Certain points should be noted about Table 14.

DIARRHOEA.

It will be seen that in 47 instances there was a history of diarrhoea. Most Indian soldiers have diarrhoea at some time, but where it is recorded in Table 14 as having been present, the diarrhoea was a definite feature of the illness, lasting for several weeks. Stool investigations did not indicate that either amoebic or bacillary dysentery was a feature in these cases. Where there was diarrhoea, the appearance of the stool varied. Sometimes it was pale, frothy and bulky as in classical sprue: at other times the motions were merely watery. Microscopically, fatty acid crystals and fat globules were occasionally seen, and sometimes undigested food was present. Blood and mucus were not found. Facilities for estimating stool fats were not available.

MALARIAL PARASITES.

It will be seen that in 42 instances malaria parasites were found in the peripheral blood either at the transit hospital or before the man reached it. This was undoubtedly an underestimate of the prevalence of malaria since the men had been under orders to take suppressive mepacrine at their units and were supposed to be having it in the forward hospitals. The administration of suppressive mepacrine was not always as regularly carried out in hospital as it should have been.

The regular administration of mepacrine (0.1 G. daily) prevented the occurrence of malaria. In the case of benign tertian malaria this was a suppressive action in that infection took place but clinical features of malaria did not develop. Indeed, at one stage in the campaign, a man who developed malaria/

malaria was in danger of being court martialled for not having taken his mepacrine.

However, suppressive mepacrine was purposely not given at Sirajgunj or Dacca to the patients shown in Table 14, and malaria parasites were frequently found in the peripheral blood, particularly after the administration of liver injections. It was thought that these crude liver injections were acting as a form of 'shock' therapy. A few experiments were carried out with sterile milk or T.A.B. injections, but malaria did not supervene, and in any event the mere cessation of the administration of suppressive mepacrine sometimes led to the appearance of parasites in the peripheral blood.

It will be seen in Table 14 that 21 patients with no proof of the presence of malaria had persistent fever and that 28 had a previous history of malaria. The spleen was palpable or tender in 14 cases without proven malaria and was <u>not</u> palpable or tender in 20 cases who had had parasites in the blood in the course of the present illness.

Sirajgunj and Dacca were not highly malarious areas, but mosquito nets were used.

<u>WEIGHT LOSS</u>.

This could not be assessed accurately. Scales were not available, but where the records gave any information about weight this is noted in Table 14. In the column 'Weight Loss' there is noted the present author's estimate from examination of the patient. This is, of course, only an extremely rough indication.

<u>ANKYLOSTOME OVA</u>.

In/

In 27 cases ova were found. All these men had had at least one stool examined and in some the stay in the Transit Hospital was sufficiently long for repeated examinations to be done. It can be taken as likely that this was an underestimate of the incidence of ankylostomiasis.

FOOD.

Most of the men were meat eaters. It is true,that the Indian soldier is not given to grumbling about his diet, and it will be seen from Table 14 that almost every man said that the food at his unit in the fighting area or jungle was good. This does not eliminate the possibility that in some instances it was, in fact, nutritionally shocking, but it seems unlikely that all these men could be suffering from primary malnutrition without being aware of the fact. Patient No.22 (Table 14) had in his records a detailed account of the diet in his unit, and it tallied almost exactly with the authorised ration scale. The basic ration for Indian soldiers contained 4,200 calories, with 120 G. of protein (26 G. of animal protein), 2.8 mg. of aneurine, 1.7 mg. of riboflavin and 75 mg. of ascorbic acid. However, supply difficulties were considerable in jungle fighting and there can be no doubt that the authorised ration scales were frequently not attained, despite all that was done by means of the parachute dropping of rations and equipment. One complicating factor was that in some instances the patients had been unable to eat their rations because they had felt feverish and ill, possibly because of malaria.

TREATMENT.

Before these men were seen in the course of the present survey/

survey, many had been treated for varying periods and at forward hospitals with high protein diet (if they could eat it), iron, vitamins, in many cases autolysed yeast preparations which were usually given in a dosage of oz. ½ t.i.d., crude liver injections, liquid liver extract by mouth, and sometimes blood transfusions. The liver injections were usually of the crude Indian liver preparation "TCF" which had been tested on pernicious anaemia cases in Britain and found to be satisfactory so far as the batches tested were concerned. Antimalarial treatment had usually consisted of mepacrine in a dosage of 0.6 G. daily for two days, 0.3 G. for five days and 0.1 G. daily thereafter, but sometimes this was supplemented by quinine, and occasionally pamaquine. In many instances the treatment recorded in Table 14 is what had already been given before the present survey took place. Where this is so the abbreviation 'Prev.' precedes the note about therapy. If no indication is given about response to previous therapy this is because active efforts at treatment had not taken place. Where treatment was given in the course of this survey the substances used were:-

Hepastab. A crude liver extract supplied by Messrs. Boots from a batch which had been clinically tested in pernicious anaemia. This was usually given in a dosage of 8 ml. daily for two days, then 4 ml. daily for 7 days.

TCF Liver Extract. A crude liver extract of Indian manufacture. This was given in a similar dosage.

Antimalarial Therapy (AMT). Two courses of treatment were tried/

tried. AMT 1 consisted of mepacrine only, given in a dosage of 0.6 G. daily for two days, 0.3 G. daily for five days, and 0.1 G. daily thereafter. AMT 2 consisted of quinine gr. XXX for two days, mepacrine 0.3 G. daily for five days, followed by two days rest then pamaquine 0.03 G. daily for five days.

ICTERUS OF THE SERUM.

This was recorded where it was obvious to the naked eye. It will be seen that it was found infrequently and not only where malaria parasites were obtained from the blood.

MARROW.

It will be seen from Table 14 that in many instances the marrows contained megaloblasts. Marrows of untreated pernicious anaemia patients were available for comparison. Representative differential marrow counts are given in Table 15. Proerythroblasts were frequently less numerous than would be expected in a patient with pernicious anaemia of equivalent degree. Intermediate megaloblasts were similar to those of pernicious anaemia except that the cytoplasm was frequently more blue, possibly because of associated iron deficiency. There was no doubt however that megaloblasts were present in many cases (see photograph, p. 99). Since intermediate megaloblasts are the most easily recognisable ones, it is the percentage of these that is given in Table 14, and their number is expressed as a percentage of the total red cell precursors. The leuco-erythroblastic ratio was low, especially where anaemia was severe. Malaria parasites were never found in the marrow where they were not obvious in the peripheral blood./

TABLE 15a. DIFFERENTIAL MARROW COUNTS ON SOME OF THE CASES RECORDED IN TABLE 14.

TABLE 15a. DIFFERENTIAL MARROW COUNTS ON SOME OF THE CASES RECORDED IN TABLE 14.

Case number	1	2	3	4	5	6	7	9	11	12	13	14	15	16
Blood:														
Haemoglobin G.%	2.8	2.9	3.7	5.1	2.9	5.3	5.2	5.2	6.3	5.8	5.1	5.8	6.8	4.6
R.B.C. (mills.)	0.395	0.835	0.864	1.02	1.09	1.19	1.26	1.29	1.32	1.33	1.41	1.41	1.43	1.44
W.B.C.	1,800	3,100	1,200	4,000	3,000	2,100	2,400	5,400	4,400	3,800	2,100	8,200	3,500	2,400
Polymorphs %	67	29	60	66	45	27	44	55	40	26	9	77	59	57
Lymphocytes	25	60	38	28	53	62	48	45	46	69	86	20	35	40
Monocytes	1	4	2	2	2	5	4	-	5	5	5	2	2	1
Eosinophils	4	7	-	4	-	6	4	-	9	-	-	1	4	2
Basophils	3	-	-	-	-	-	-	-	-	-	-	-	-	-
P.C.V. %	8.0	11.0	12.0	14.6	9.1	16.4	16.0	17.3	21.0	17.0	16.3	18.5	19.7	*
M.C.H.	70.9	34.7	42.8	49.9	26.6	44.5	46.8	40.3	47.7	43.6	36.2	41.0	47.6	32.1
M.C.H.C. %	35.0	26.4	30.8	34.9	31.9	32.3	36.9	30.0	30.0	34.1	31.3	31.4	34.5	-
M.C.V. (cu.)	177.0	131.7	138.9	142.7	83.4	137.8	127.0	134.1	159.1	127.8	115.6	130.9	137.2	-
Reticulocytes %	3.75	1	< 1	2.75	< 1	< 1	< 1	< 1	2.75	1.25	2.0	1.75	2.75	1.5
Marrow:														
Neutrophils														
Myelocytes	2.0	-	-	0.25	2.5	1.75	-	-	-	1.25	-	2.5	-	1.0
Metamyelocytes	1.75	14.25	1.75	7.25	6.25	3.5	8.25	7.5	10.5	4.25	4.0	13.5	12.5	6.0
Polymorphs	20.25	30.25	51.25	28.25	46.75	9.0	28.75	29.75	9.5	24.5	19.75	47.75	36.0	38.25
Eosinophils														
Myelocytes	0.75	0.5	-	-	-	0.25	0.75	-	1.0	0.25	1.5	0.5	0.5	1.0
Polymorphs	2.25	2.0	0.75	1.0	1.25	0.75	0.25	0.5	2.0	0.75	0.75	0.75	2.0	3.0
Basophils														
Myelocytes	-	-	-	-	-	-	-	-	-	-	-	-	0.75	-
Polymorphs	-	-	-	-	-	-	-	-	-	0.25	-	0.25	0.25	-
Premyelocytes	0.5	-	0.75	-	1.5	0.75	-	-	0.75	0.5	-	0.25	-	0.25
Myeloblasts	3.25	-	1.0	-	2.25	2.0	0.75	-	0.25	3.5	-	0.25	-	0.5
Lymphocytes	5.75	3.75	-	4.75	3.5	1.75	0.5	-	1.5	0.25	0.5	0.5	-	3.25
Plasma cells	-	-	-	-	0.5	-	0.25	-	-	0.5	-	-	-	-
Monocytes	-	-	-	-	-	-	-	-	-	-	-	-	1.5	-
Megakaryocytes	-	-	-	-	0.25	-	-	-	-	-	-	-	-	-
Proerythroblasts	3.0	1.75	-	0.5	7.25	5.75	0.25	2.5	7.0	20.5	6.5	-	2.0	5.75
Early normoblasts														
Normal	7.5	2.0	3.0	2.5	10.75	4.5	14.25	14.75	5.75	11.25	17.0	4.0	3.25	7.0
Hypochromic	7.5	2.5	-	-	0.25	1.25	-	-	-	1.0	1.25	1.5	1.0	-
Early megaloblasts	0.75	1.5	-	2.5	0.75	9.5	1.25	0.25	1.5	5.0	2.75	0.75	1.75	5.25
Intermediate normoblasts														
Normal	2.5	3.75	4.5	1.0	4.0	14.5	25.0	8.5	5.75	4.75	12.0	2.25	3.25	7.0
Hypochromic	17.75	3.5	-	-	0.5	1.5	-	-	0.5	1.75	-	8.5	3.0	1.0
Intermediate megaloblasts	7.25	10.0	6.5	20.5	1.0	6.25	1.0	1.0	30.5	13.5	8.0	2.25	10.5	9.0
Late normoblasts														
Normal	-	13.0	28.75	-	7.5	25.0	17.0	29.75	6.25	-	17.25	1.75	5.75	6.75
Hypochromic	5.5	4.75	-	-	0.25	1.5	-	3.5	-	2.0	3.5	7.5	9.25	0.75
Late megaloblasts	11.75	6.5	1.75	31.5	3.0	10.5	1.75	2.0	17.25	4.25	5.25	5.25	6.75	4.25

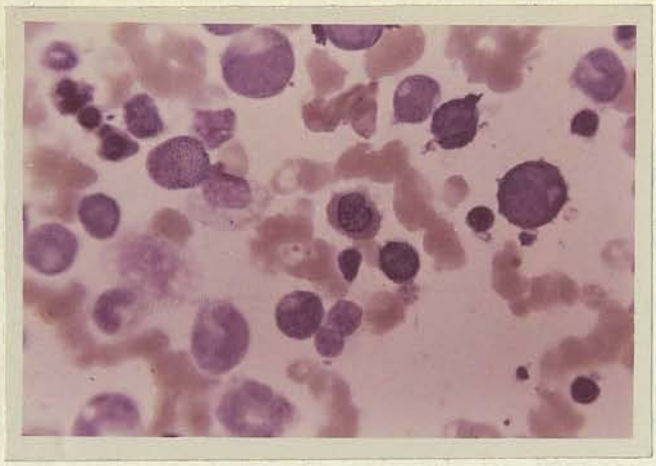
* Haematocrit examinations carried out a few days later in these cases showed the anaemia to be orthochromic and macrocytic.

TABLE 15b. DIFFERENTIAL MARROW COUNTS ON SOME OF THE CASES RECORDED IN TABLE 14.

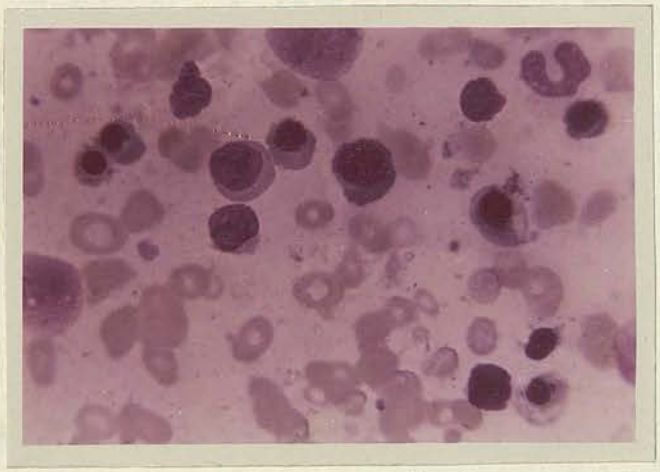
TABLE 15b. DIFFERENTIAL MARROW COUNTS ON SOME OF THE CASES RECORDED IN TABLE 14.

Case number	17	21	25	29	31	33	35	39	40	43	44	56	76	78	79	P A**
Blood:																
Haemoglobin G.%	5.2	6.9	6.1	7.4	8.6	8.1	8.0	10.0	8.4	8.5	10.0	12.2	12.6	6.5	8.8	9.2
R.B.C. (mills.)	1,46	1.68	1.81	2.01	2.08	2.21	2.251	2.37	2.38	2.53	2.55	3.00	3.56	3.61	3.62	2.23
W.B.C.	1,600	4,700	3,800	4,800	4,500	2,300	9,100	3,000	3,200	3,800	3,800	5,700	2,200	3,100	4,500	-
Polymorphs	41	71	40	44	58	46	33	49	51	56	56	42	39	38	56	-
Lymphocytes	57	29	59	49	28	51	58	46	40	38	44	47	54	58	31	-
Monocytes	-	-	2	2	4	1	-	3	4	3	-	4	4	2	4	-
Eosinophils	2	-	3	5	10	2	9	2	5	3	-	7	3	-	9	-
Basophils	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-
P.C.V. %	15.2	20.4	18.1	27.5	*	23.3	26.2	28.2	24.9	31.0	27.0	38.0	41.0	25.0	29.0	-
M.C.H.	35.6	41.1	33.7	36.8	41.3	36.7	35.6	42.2	35.3	33.6	39.3	40.1	35.4	18.0	24.3	41.7
M.C.H.C. %	34.0	33.8	33.7	26.9	-	34.8	30.5	35.5	33.7	27.4	36.3	32.1	30.7	26.0	30.3	-
M.C.V. (cu.)	103.8	121.8	100.0	137.3	-	105.4	116.7	119.0	104.6	122.5	105.9	125.0	115.2	69.3	80.1	-
Reticulocytes %	< 1	< 1	< 1	1.0	8.25	< 1	6.75	< 1	< 1	< 1	< 1	< 1	2.5	2.5	< 1	-
Marrow:																
Neutrophils																
Myelocytes	0.5	-	-	0.75	0	1.5	0.5	0.75	-	-	-	0.25	-	0.5	1.75	-
Metamyelocytes	10.0	11.0	7.0	13.0	27.0	16.25	13.5	12.5	6.75	1.75	4.75	7.5	14.0	11.5	24.5	3.75
Polymorphs	37.5	45.5	48.25	54.5	47.5	32.5	31.0	39.5	26.0	7.5	35.0	65.5	31.75	38.5	37.5	28.25
Eosinophils																
Myelocytes	-	-	0.75	0.25	0.5	-	0.25	3.25	0.5	0.25	-	-	-	-	3.5	-
Polymorphs	1.0	0.5	4.0	3.25	0.25	0.75	3.0	3.5	2.25	1.25	0.25	6.5	3.75	-	5.5	0.75
Basophils																
Myelocytes	-	0.25	-	-	-	-	-	-	-	-	-	-	-	-	0.25	-
Polymorphs	0.25	-	0.5	0.25	-	-	1.0	-	-	-	-	-	0.25	-	0.75	-
Premyelocytes	-	-	-	-	-	0.75	0.75	-	0.25	0.5	-	-	-	0.25	1.0	-
Myeloblasts	0.5	-	0.25	-	-	2.0	1.0	1.75	2.75	2.5	-	-	0.5	1.5	1.25	0.25
Lymphocytes	1.0	-	1.5	1.0	-	-	0.25	0.25	-	-	0.5	-	1.5	0.25	3.0	3.75
Plasma cells	1.0	-	-	0.25	-	-	-	0.25	0.75	-	-	0.25	1.5	-	0.25	-
Monocytes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.5	-
Megakaryocytes	-	-	-	0.25	-	-	-	-	-	-	-	-	0.5	-	0.5	-
Pro-erythroblasts	-	1.25	2.0	1.5	-	1.5	5.0	1.75	-	-	-	0.5	0.25	1.0	1.5	3.25
Early normoblasts																
Normal	9.75	8.0	3.5	3.75	1.5	2.5	9.5	12.0	10.5	10.75	1.0	0.5	4.0	2.75	3.25	0.25
Hypochromic	-	3.25	4.25	-	-	5.0	0.5	4.0	-	-	-	0.5	3.0	0.75	-	-
Early megaloblasts	2.75	0.5	2.25	0.25	-	1.25	-	0.5	-	-	-	-	-	-	-	9.75
Intermediate normoblasts																
Normal	8.25	2.25	1.75	7.0	6.5	0.5	16.0	2.5	25.0	36.25	6.25	3.5	5.5	4.25	4.75	6.75
Hypochromic	-	5.75	3.75	0.25	-	3.0	1.25	5.75	-	-	3.0	1.25	10.5	13.5	-	1.0
Intermediate megaloblasts	8.25	3.25	8.5	3.25	-	5.0	-	2.75	1.25	1.5	-	2.75	0.5	-	-	13.5
Late normoblasts																
Normal	14.75	8.5	1.75	7.75	16.75	0.5	15.5	1.75	21.25	36.25	46.5	7.5	8.0	-	8.25	16.5
Hypochromic	-	8.75	5.25	1.5	-	22.75	0.5	3.0	-	-	2.75	3.0	14.0	25.25	1.0	5.25
Late megaloblasts	4.5	1.25	4.75	1.25	-	4.25	0.5	4.25	2.75	1.5	-	0.5	0.5	-	-	8.0

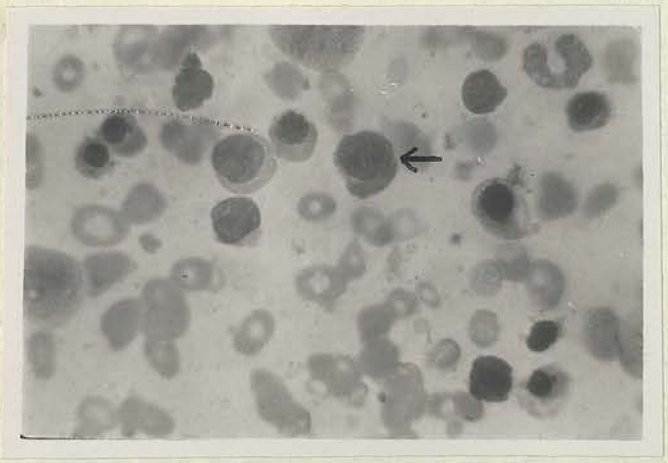
** A Marrow of an untreated pernicious anaemia case obtained from Britain and used at the time for reference purposes.



The bone marrow of Case 4 (also Graph 4). See pp.102 & 110. An intermediate megaloblast (sometimes called polychromatic megaloblast) is seen near the centre of the field.



The bone marrow of Case 20 (see Table 14a).



Key to colour picture of marrow of Case 20. An arrow points to an intermediate megaloblast. As was frequently the case the cytoplasm of this cell stained with a more blue colour than is usually found in the cytoplasm of the intermediate megaloblasts in untreated pernicious anaemia. Leishman's stain was used.

blood.

* BLOOD COUNTS AND FILMS.

The blood was venous and was drawn, without stasis, into Wintrobe's dry oxalate mixture. It will be seen from Table 14 that macrocytosis was frequently present and that reticulocytes were not sufficiently increased to be the main cause of this macrocytosis. The peripheral blood of patients with severe megaloblastic anaemia was similar to that of untreated pernicious anaemia patients, showing anisocytosis, poikilocytosis, ovalocytosis and, if the anaemia was severe, the presence of normoblasts in the peripheral blood. Arneth counts were carried out on the neutrophil polymorphs in all these patients — a shift to the right did not occur even with a frankly megaloblastic marrow.

NOTES ABOUT REPRESENTATIVE CASES.

Although a summary of the main features of a number of the cases investigated is included in Tables 14 and 15, it is necessary to give further information about representative cases to show the possible interplay of factors that could lead to anaemia, and the varying responses to therapy. For this purpose the following fourteen cases have been selected. Two of them do not appear in Tables 14 or 15.

Case/

---

* The blood counts and marrow findings in Table 14 are those after any treatment marked 'Prev.' but before any marked 'Now'.

Case 44. In Table 14 there are included the particulars of one British soldier, a private aged 27 years with 4½ years service in an Infantry unit. He had been in Assam or Burma for six months and had been taking 0.3 G. of suppressive mepacrine daily for five months, then 0.1 G. daily thereafter. His skin was stained yellow with mepacrine. The food at his unit had been entirely satisfactory, and there was no previous history of malaria. He had had typhoid fever several years previously. The symptoms necessitating admission to hospital were of fever and slight diarrhoea without the passage of blood or mucus. He had lost some weight. Suppressive mepacrine was stopped and the parasites of benign tertian malaria appeared in the blood after twelve days. At this time the blood figures were Hb. 10.0 G. per 100 ml., red cells 2,546,000 per c.mm., PCV 27.0%, MCV 105.9 cμ., MCHC 36.3%, WBC 3,800, Retics. < 1%. As will be seen from Graph 1, the anaemia persisted despite antimalarial therapy, and the parasites of BT malaria appeared in the blood on numerous occasions. The marrow was normoblastic. No diarrhoea occurred in hospital and there was no splenic enlargement or glossitis. He did not suffer from ankylostomiasis.
Summary. A British patient with chronic relapsing malaria and macrocytic anaemia, apparently due to this. The anaemia which was normoblastic did not respond satisfactorily to antimalarial therapy. (See Graph p.109).

Case 98. Although severe anaemia was not common in British troops in Burma it did occur. Mention should perhaps be made of one such patient whose particulars are not included in Table 14 because he had been treated successfully before he was seen in the course of the present investigation. He was a private in an Infantry Regiment and was aged 21 years. His unit food had been good and there was no history of diarrhoea or sore tongue. Of his three years service, two months had been spent in Assam where he was supposed to be having suppressive mepacrine. In December 1944 he developed benign tertian malaria which was treated with mepacrine. Seven days after the commencement of treatment for this a blood count was done and the haemoglobin level was found to be 3.9 G. per 100 ml., red cells 1,290,000 per c.mm., MCH 30.4 Despite the continuation of mepacrine therapy, malaria parasites persisted in the peripheral blood and the anaemia became more severe, the red cell count falling to 980,000 per c.mm. Mepacrine therapy was continued, together with liquid extract of liver by mouth, the crude liver preparation Hepalon intramuscularly, and transfusion of a pint of blood. The temperature subsided and when we first saw the patient, two months after the onset of the illness and five weeks from the time when the counts were lowest, the haemoglobin level was 13.1 G. per 100 ml., red cells 4,665,000 per c.mm., PCV 44%/

44%, MCV 94.3 cμ. The spleen which had been enlarged was no longer palpable. There was no glossitis.
Summary. A British soldier who had developed severe anaemia from benign tertian malaria, but who responded to antimalarial therapy supplemented by transfusion and liver injections.

Case 9. This was an Indian sapper aged 25 years who had served for over four years. Three years had been spent in Iraq and nearly one year in Assam. He was admitted to hospital in October 1944 with a four months history of weakness, diarrhoea and painful tongue, and when he was seen by the author in December 1944, the diarrhoea continued although he had had 26 G. of sulphaguanidine. There was no blood or mucus in the stool which was pale, bulky and offensive with undigested meat fibres but no evidence of intestinal parasites. The patient, who was a vegetarian, considered that the food supplied at his Unit was satisfactory. There was no previous history of importance. The patient had been having 0.1 G. of mepacrine daily at his Unit, and so far as he knew he had never had malaria. He was emaciated, weighing only 80 lbs., and the tongue was reddened and atrophic. The spleen was not palpable and no malaria parasites were found in the peripheral blood. The haemoglobin level was 5.2 G. per 100 ml., red cells 1,290,000 per c.mm., PCV 17.3%, MCV 134.1 cμ., MCHC 30.0%. Of the red cell precursors in the marrow, 4 per cent were proerythroblasts, 0.5 per cent early megaloblasts, 1.7 per cent intermediate megaloblasts and 3.2 per cent late megaloblasts. This was despite six weeks treatment with iron, autolysed yeast in a dosage of one teaspoonful t.i.d., and liquid liver extract by mouth in a dosage of oz. 1, t.i.d. The patient was evacuated at once to a base hospital.
Summary. An Indian patient with clinical features of tropical sprue and megaloblastic anaemia, despite treatment with autolysed yeast and the oral administration of a liquid liver extract. Malarial infection had not been demonstrated.

Case 21. A sepoy with four years service who had spent two years in Assam. There was a previous history of three attacks of malaria. For some reason he had never received suppressive mepacrine. He was a vegetarian who said that the food supplied at his Unit was satisfactory, but that for six months before admission to hospital he had been unable to eat because of fever. His main complaints now were of weakness, giddiness and loss of appetite. There had been no diarrhoea previously but now, after a month in various hospitals it had developed, and the stool contained hookworm ova. A month before he was seen in the course of the present investigation he had been found to have benign tertian malaria and had been treated/

treated for it with mepacrine. The spleen was not palpable. There appeared to be considerable loss of weight and the tongue was commencing to be painful. As is shown in Table 14 the haemoglobin level was 6.9 G. per 100 ml., red cells 1,680,000 per c.mm. The PCV was 20.4%, and the MCV 121.8 cμ. In the marrow 7.6% of the red cell precursors were intermediate megaloblasts. The course of the illness from the haematological aspect is shown in Graph 2. It will be seen that on two occasions the parasites of malignant tertian malaria appeared in the peripheral blood in the course of treatment with liver injections, and that after about eight weeks from the commencement of the present investigation improvement occurred in the blood figures. At the same time the appetite improved, diarrhoea ceased and the glossitis, which had by now appeared, subsided.

Summary. An Indian with megaloblastic anaemia. There was malignant tertian malarial infection and it was possible that a complication was primary malnutrition due to anorexia caused by the fever.

Case 11. A sepoy aged 22 years who had nearly three years of service. He was a high caste Brahmin and a vegetarian. He had spent eight months east of the Brahmaputra river at Chittagong, then outwith the active fighting zone. The food supplied to him at his Unit was plentiful and satisfactory. His complaints were of weakness, giddiness and anorexia with no diarrhoea. Fever was intermittent. There was a previous history of ten attacks of malaria and he had received mepacrine regularly at Chittagong. There was no weight loss, the spleen was enlarged to five fingers' breadth and the stool, which appeared normal, contained ova of ankylostome. The tongue was slightly painful, but appeared normal. As can be seen in Table 14 the haemoglobin level was 6.3 G. per 100 ml., red cells 1,320,000 per c.mm., PCV 21%, MCV 159.1 cμ., MCHC 30.0%. The serum was icteric. The marrow contained a higher percentage of megaloblasts than did that of any other case in the series (see Table 15). As is shown in Graph 3, the patient was given three injections of Hepastab and parasites of benign tertian malaria promptly appeared in the peripheral blood. The malaria was treated and the blood figures rose rapidly to normal.

Summary. A vegetarian Indian patient with recurrent malaria and severe megaloblastic anaemia which responded to liver injections followed by antimalarial therapy.

Case 4. A lance-naik (lance corporal) aged 23 years who had served for four years in a Provost Unit. He had been in Burma for five months and in Assam for four months before that. He gave no previous history of illness; there was no glossitis, his appetite was fairly good, the bowels were normal, the spleen was not enlarged, and the stool contained no ova of ankylostome. His complaint was of/

of breathlessness of about seven weeks duration. The patient, who was a meat eater, considered that the food at his Unit had been entirely satisfactory, meat and eggs being provided. He had received 0.2 G. of mepacrine daily at his Unit and also in the forward hospital from which he had just been admitted. As can be seen from Tables 14 and 15 there was severe megaloblastic anaemia, the haemoglobin level being 5.1 G. per 100 ml., red cells 1,023,000 per c.mm., PCV 14.6%, MCV 142.7 cμ., MCHC 34.9 %. No malaria parasites were found at any time in the peripheral blood. As will be seen in Graph 4 there was a rapid response to injections of liver extract, the administration of mepacrine being continued simultaneously in a higher dosage.

Summary. An Indian patient with severe megaloblastic anaemia and with no definite evidence of primary malnutrition, sprue, ankylostomiasis or malaria, who responded to liver injections. Antimalarial therapy was also administered since mepacrine had been given continuously for ten months.

Case 16. A sepoy aged 18 years, with one and a half years' service. He had served in Chittagong for four months and had taken no part in jungle warfare. His symptoms were of breathlessness and palpitation of six months duration. There was no diarrhoea, and the food supply at his Unit had been good. The patient was a vegetarian. There was a previous history of malaria a year previously. The stools were normal and did not contain ankylostome ova, and the spleen was not palpable. There was no glossitis or obvious weight loss. When first seen in the course of this investigation the patient had already been treated with mepacrine for malignant tertian malaria which had manifested itself seventeen days previously. As can be seen from Tables 14 and 15, the marrow was megaloblastic and the haemoglobin level was 4.6 G. per 100 ml., red cells 1,435,000 per c.mm. despite this therapy. (The centrifuge was broken at the time, but the anaemia was later shown to be orthochromic and macrocytic.) The patient was treated with Hepastab and showed a response to this, as demonstrated in Graph 5.

Summary. An Indian patient with megaloblastic anaemia following malignant tertian malaria. There was a response to treatment with liver injections.

Case 29. An Indian driver aged 30 years who had spent $1\frac{1}{2}$ years of his $2\frac{1}{2}$ years service in Assam. He had reported sick after having had diarrhoea, flatulence, weakness and headache for about three months. There was a previous history of malaria six months previously. The patient was a meat eater and stressed that the food at his Unit had been very good, meat being plentiful. Suppressive mepacrine had been taken. The stools were pale and watery and contained meat fibres and fat globules. There appeared/

appeared to have been some weight loss and the spleen was palpable, but the tongue showed only slight atrophic changes in the papillae. This patient had been treated as follows before the present author first saw him:-

Days 1 - 12	2 ml. TCF liver extract daily IM.
30 - 37	2 ml. TCF liver extract daily IM
28 - 46	Liquid liver extract oz. 1 t.i.d. by mouth.
22 - 26	Sulphaguanidine. 50 G. given.
1 - 46	Ferri et ammon cit., ascorbic acid and nicotinic acid given.
31	Parasites of benign tertian malaria found in the peripheral blood and antimalarial therapy commenced. (Quinine, mepacrine and pamaquine).

The sternal puncture was carried out on the 54th day, and the marrow found to be megaloblastic. Blood counts had been done regularly and it was obvious that the anaemia had shown no response to treatment, the haemoglobin level being 7.4 G. per 100 ml., red cells 2,010,000 per c.mm., PCV 27.5%, MCV 137.3 cμ., MCHC 26.9%. The patient was promptly evacuated to a base hospital.

Summary. A patient who did not suffer from recent primary malnutrition but who had benign tertian malaria and a sprue like condition. The anaemia had not responded to liver injections or antimalarial therapy, the marrow remaining megaloblastic.

Case 25. A sepoy aged 25 who had spent two years of his two and a half years' service in Assam. He had reported sick on account of weakness and loss of appetite, with pain in the tongue but no diarrhoea. There was a previous history of two attacks of malaria. The patient was a vegetarian who claimed that the food at his Unit was plentiful. He had been taking suppressive mepacrine. The stools contained no ankylostome ova, and were otherwise normal. The spleen was not palpable and malaria parasites were not found at any time in the peripheral blood. The tongue appeared normal. When the patient was first seen in the course of the present investigation he had received intensive treatment including TCF liver injections, autolysed yeast, fresh liver by mouth and blood transfusion. Nevertheless the anaemia was severe, the haemoglobin level being 6.1 G. per 100 ml., red cells 1,810,000 per c.mm., PCV 18.1%, MCV 100.0 cμ., MCHC 33.7%, and the marrow was frankly megaloblastic. Of the red cell precursors, proerythroblasts were 5.2%, early megaloblasts 6.0%, intermediate megaloblasts 22.6% and late megaloblasts 12.6%. In Graph 6 there is shown the slow response that eventually occurred with liver injections and antimalarial therapy; the counts done before the present investigation are indicated as they had been carefully done by two officers who were particularly interested in the case.

Summary./

Summary. An Indian patient with frankly megaloblastic anaemia despite the administration of autolysed yeast, fresh liver by mouth and liver injections. The precise course of the anaemia was not evident, but there was eventual improvement with further liver injections and antimalarial therapy.

Case 14. A labourer in the Pioneer Corps aged 37, who had spent six months of his three and a half years service in Assam. He complained of the symptoms of anaemia, with loss of weight and fever. There was no diarrhoea. The previous history was of two attacks of malaria and one of pneumonia. He had been having suppressive mepacrine at his Unit and he considered that the food there was satisfactory. He was a meat eater. There was evidence of some weight loss, the tongue was normal, the spleen was not palpable and the stools were normal and did not contain ova of ankylostome. Slight fever was present. He had been under observation without treatment for a month, and had remained anaemic. The blood counts were haemoglobin 5.8 G. per 100 ml., red cells 1,413,000 per c.mm., PCV 18.5%, MCV 130.9 cμ., MCHC 31.4%. At this time the reticulocyte percentage was 1.75 but the serum was not icteric. The marrow was megaloblastic. No treatment was given for sixteen days, and as can be seen in Graph 7 there was a slow improvement in the blood counts and clinical condition, icterus becoming evident in the serum. The patient was given injections of liver extract followed by mepacrine. There was a slight increase in the percentage of reticulocytes following liver therapy but no great change in the rate of rise of the red cell count. Summary. A patient with megaloblastic anaemia who showed a spontaneous remission after a month in hospital.

Case 78. A labourer in the Pioneer Corps aged 30 years, who had spent three months of his year's service in Chittagong and had seen no active fighting. He complained of headache and giddiness with no diarrhoea or glossitis. He was a meat eater, the food at his Unit, which was in a base area, had been good and he had had regular suppressive mepacrine. He thought that he had once had malaria in the past. There was no marked weight loss and no fever. Repeated stool examinations revealed no evidence of ankylostomiasis and the spleen was not palpable. As can be seen in Table 14 the haemoglobin level was 6.5 G. per 100 ml., red cells 3,610,000 per c.mm., PCV 25%, MCV 69.3 cμ., MCHC 26.0%. The marrow contained many hypochromic late normoblasts. The centrifuge had burned out when the sternal puncture was done, and it was not until three weeks after the sternal puncture that these absolute indices could be ascertained. In that period there had been no response to ferrous sulphate gr.V t.i.d. The patient had previously been given tetrachlorethylene in case he might/

might have ankylostomiasis. The stool benzidene was negative. Suppressive mepacrine had not been administered in hospital. As can be seen in Graph 8, the injection of Hepastab was followed by the appearance of parasites of benign tertian malaria in the peripheral blood, and a rise in the red cell and haemoglobin levels. This continued when mepacrine was administered.
Summary. An Indian patient with iron deficiency anaemia which did not respond satisfactorily until liver injections were given. Malaria was a 'hidden' complication.

Case 99. This patient's particulars are not included in Table 14 since the man who was a Sikh and who could not have his very hairy chest shaved for religious reasons, would not permit a sternal puncture to be done. He was a sapper aged 30 years with six months service in Burma and two years service in India before that. He was a meat eater who considered the food at his Unit to be good. He had never had malaria or suppressive mepacrine, and his complaint was of weakness without diarrhoea, glossitis or fever. There was no evidence of weight loss, and the spleen was not palpable. The serum was not icteric. The stools contained ova of ankylostome, but despite treatment with carbon tetrachloride and ferrous sulphate gr.V t.i.d., there was no rise in the blood count in twenty-one days, the level at the commencement of treatment being haemoglobin 8.0 G. per 100 ml., red cells 3,730,000 per c.mm., reticulocytes < 1%, and at the end of three weeks, haemoglobin 7.3 G. per 100 ml., red cells 3,800,000 per c.mm., reticulocytes < 1%. The MCHC was 26.1%. (See Graph 9). The patient was given three injections of Hepastab (8 ml., 8 ml. and then 4 ml.) on successive days, whereupon fever developed and parasites of benign tertian malaria appeared in the peripheral blood. Treatment was with mepacrine, but no haematological improvement occurred in the fourteen days during which the patient remained in hospital.
Summary. A case of refractory iron deficiency anaemia with 'hidden' benign tertian malaria as a complication.

Case 5. A member of the Indian Pioneer Corps aged 28 years who had spent five months of his two years of service in the Arakan. He had reported sick on 23.5.45 with a history of flatulence, anorexia, breathlessness and occasional loose motions. There was a previous history of malaria seven months previously. The patient was a meat eater who considered that the food at his Unit was satisfactory. He had had regular suppressive mepacrine. The spleen was tender, and the stools were loose and bulky and contained ova of ankylostome. The tongue was normal. When he was first seen in the course of this investigation/

investigation on 21.6.45 he had already been treated with mepacrine, iron, carbon tetrachloride, sulphaguanidine, autolysed yeast (oz. ½ b.i.d.) and six ounces of fresh lightly cooked liver daily by mouth. These lasttwo had only been given for a week but the liver was continued during the period of the investigation. The haemoglobin level was 4.2 G. per 100 ml., red cells 1,535,000 per c.mm. The marrow contained a small proportion of intermediate megaloblasts (1.2% of the total cells). The serum was not icteric. Suppressive mepacrine was stopped, and the patient developed parasites of benign tertian malaria in the blood four days later. As will be seen from Graph 10 there was no haematological response to mepacrine or Hepastab. The marrow still showed some evidence of megaloblastic change. (The blood counts recorded in Table 14 are those after liver injections because it was at this stage that a new centrifuge was obtained). Blood transfusion was required to raise the blood level, and both diarrhoea and fever continued.
Summary. An Indian patient with normochromic normocytic anaemia with a small proportion of megaloblasts in the marrow. There was no immediate response to fresh liver by mouth, antimalarial therapy or liver injections.

Case 2. An Indian Army driver aged 26 years who had spent practically all of his three years of service in Assam. He had been sent to hospital ten weeks previously with a two months history of weakness, giddiness, loss of appetite and intermittent diarrhoea. He was a vegetarian who said that the food at his Unit was plentiful and that he had taken suppressive mepacrine regularly. He had been losing weight and recently had developed a painful tongue. The spleen had never been palpable and there had been no fever in hospital, even when he had been given adrenaline injections in an unsuccessful effort to produce malaria parasites in the peripheral blood. Suppressive mepacrine was not given in the forward hospitals since malaria parasites were being sought. There was a previous history of malaria just before the onset of the present illness. The diarrhoea was continuing despite the administration of sulphaguanidine. No ova were found in the stools which were liquid, yellowish and offensive with no microscopic abnormality. He had already been treated with iron and ammonium citrate, kaolin, autolysed yeast and adexolin and had had six daily injections of 2 ml. of TCF liver extract finishing eleven days before the present investigation began. The tongue was atrophic and furred, but not inflamed. As will be seen from Tables 14 and 15 the marrow was frankly megaloblastic, and the haemoglobin level was 2.9 G. per 100 ml., red cells 835,000 per c.mm., PCV 11%, MCV 131.7 cμ., MCHC 26.4%. The patient's condition was very serious, and transfusions were given. (Graph 11.) Injections of Hepastab were commenced, and after seven days parasites both of benign tertian/

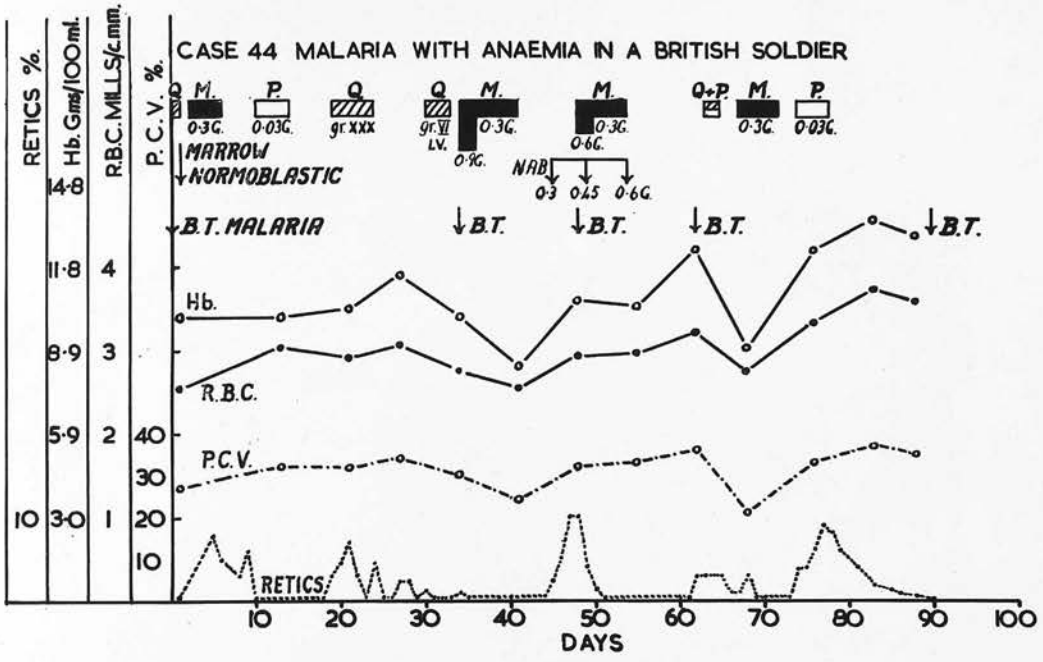
tertian and malignant tertian malaria appeared in the peripheral blood. The patient would not submit to further sternal puncture. Quinine, mepacrine and pamaquine were administered and further treatment with Hepastab and mepacrine given, but the haematological response was incomplete. There was, however, distinct clinical improvement and cessation of diarrhoea, the improvement commencing towards the end of the first course of antimalarial therapy.

Summary. An Indian patient with very severe megaloblastic anaemia associated with diarrhoea. After liver injections were given parasites both of benign tertian and malignant tertian malaria appeared in the peripheral blood. This infection was treated and thereafter improvement occurred.

DISCUSSION

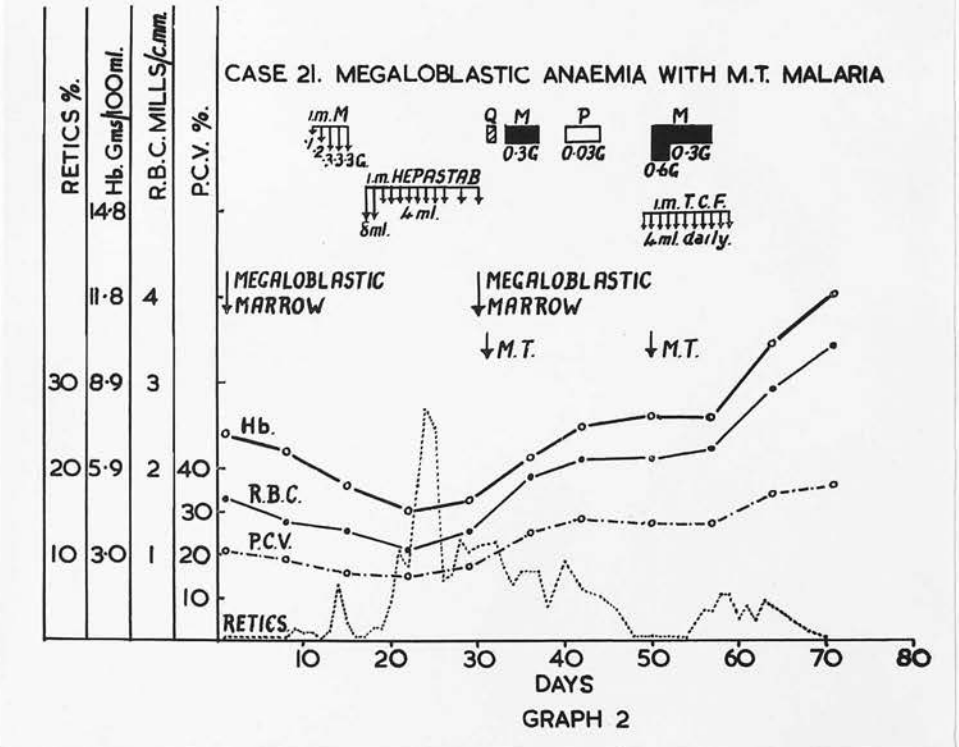
The first impression that one obtains from perusal of Tables 14 and 15 and from the representative case reports is that there is hopeless confusion. This was the impression at the Medical Directorate in Delhi and it was for this reason that the survey was carried out. It should be said at once that the problems encountered in Assam and Burma were probably much more complex than those facing Lucy Wills in Bombay or Das Gupta in Calcutta. We have already seen that the problem of macrocytic anaemia did not exist amongst similar troops stationed in areas west of Assam and Burma. In the series of cases that we have described there are some patients with megaloblastic anaemia who had clinical features of sprue, and others who have no evidence of any cause other than malaria. An occasional patient has a spontaneous remission while another does not respond to any treatment. Rarely there is definite improvement with liver injections, and frequently the injection of crude liver preparations is followed by the appearance of malaria parasites in the peripheral blood. Sometimes the

treatment/

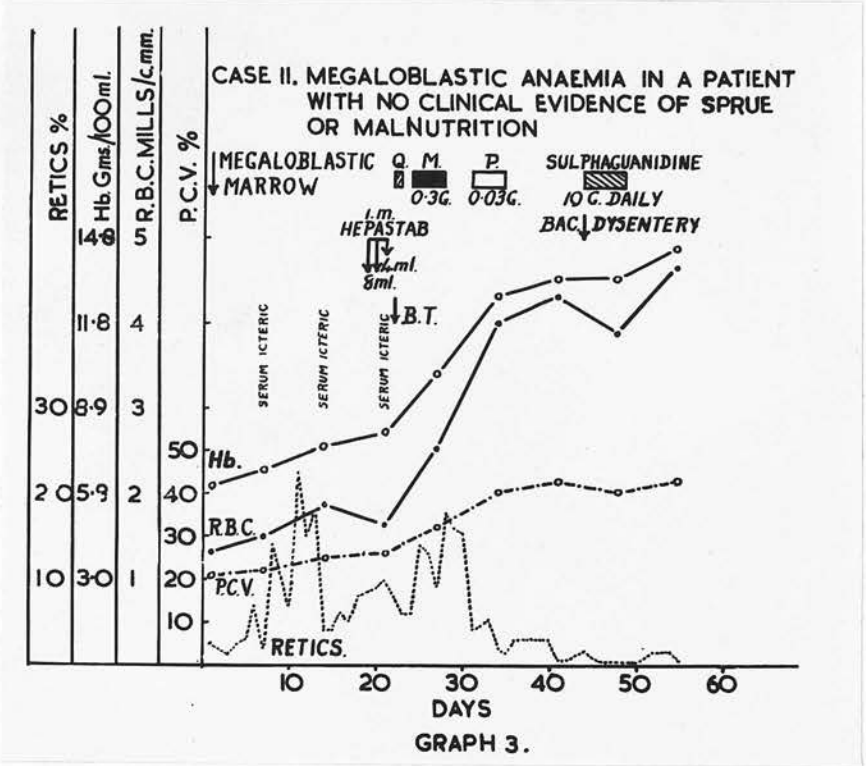


GRAPH 1.

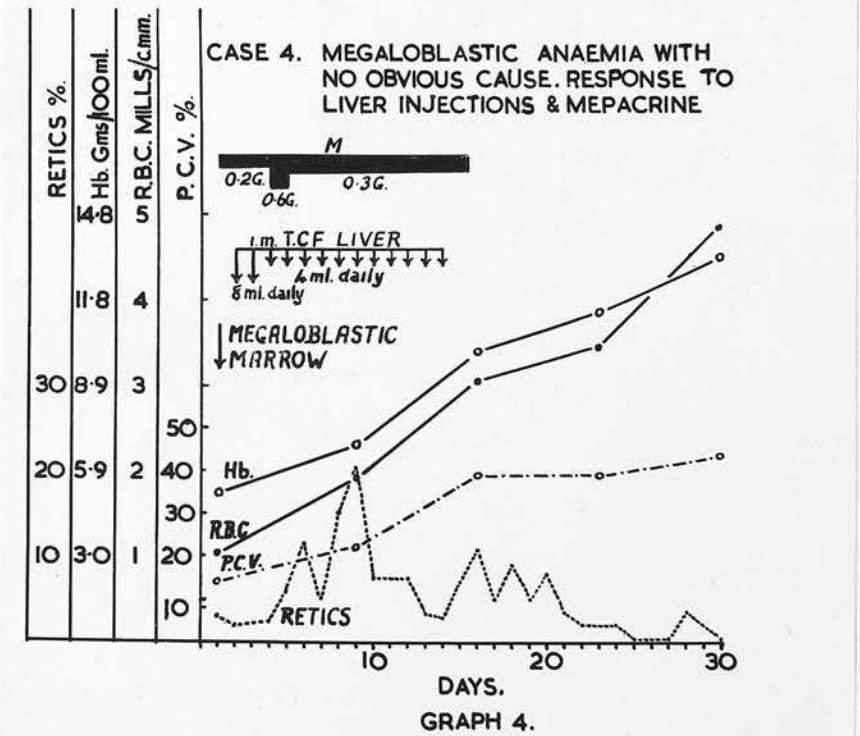
Note: Q = Quinine. M = Mepacrine. P = Pamaquin.



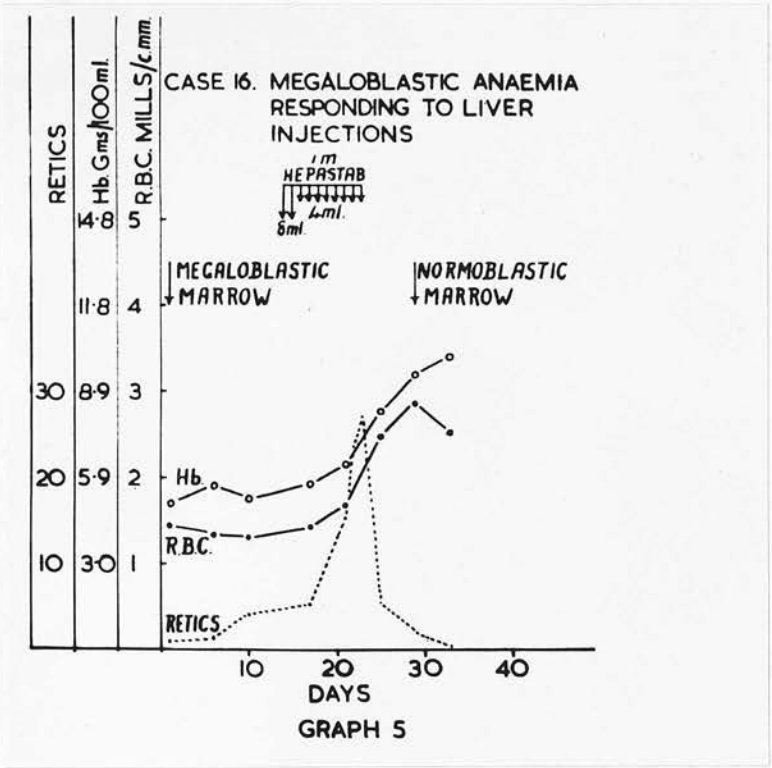
GRAPH 2



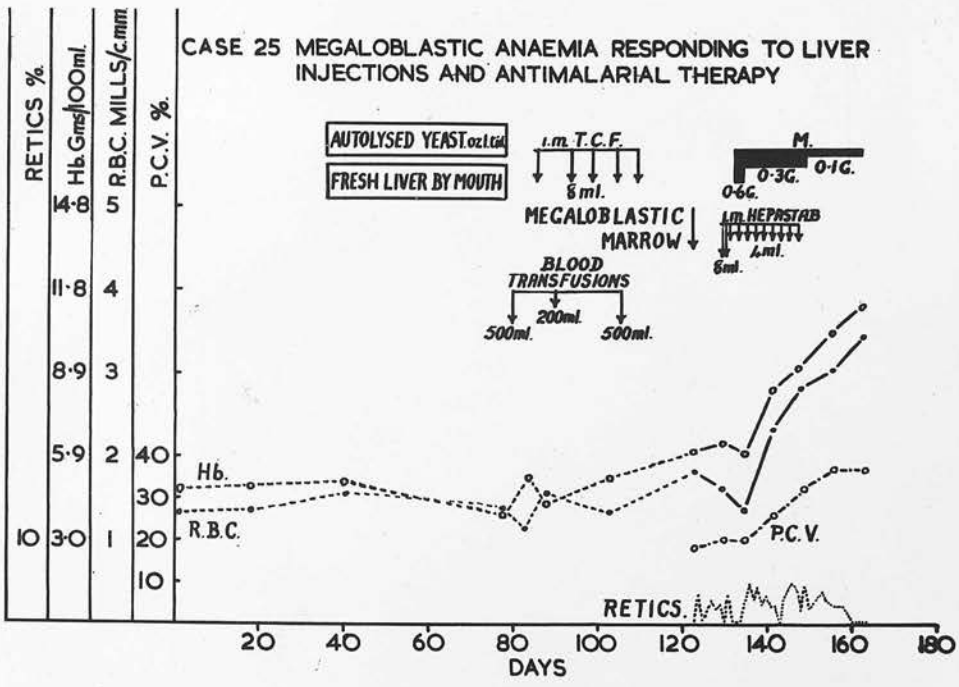
GRAPH 3.



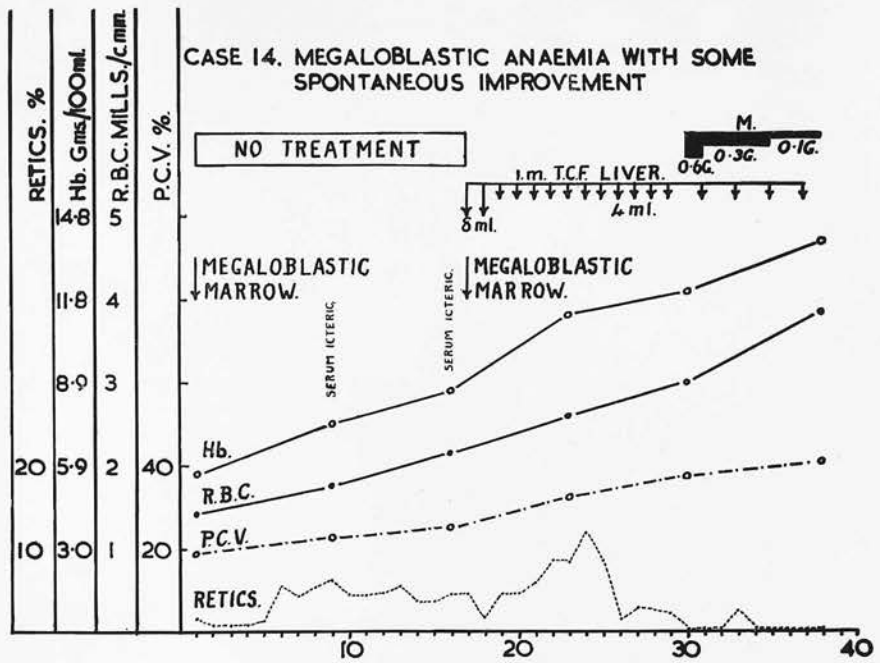
GRAPH 4.



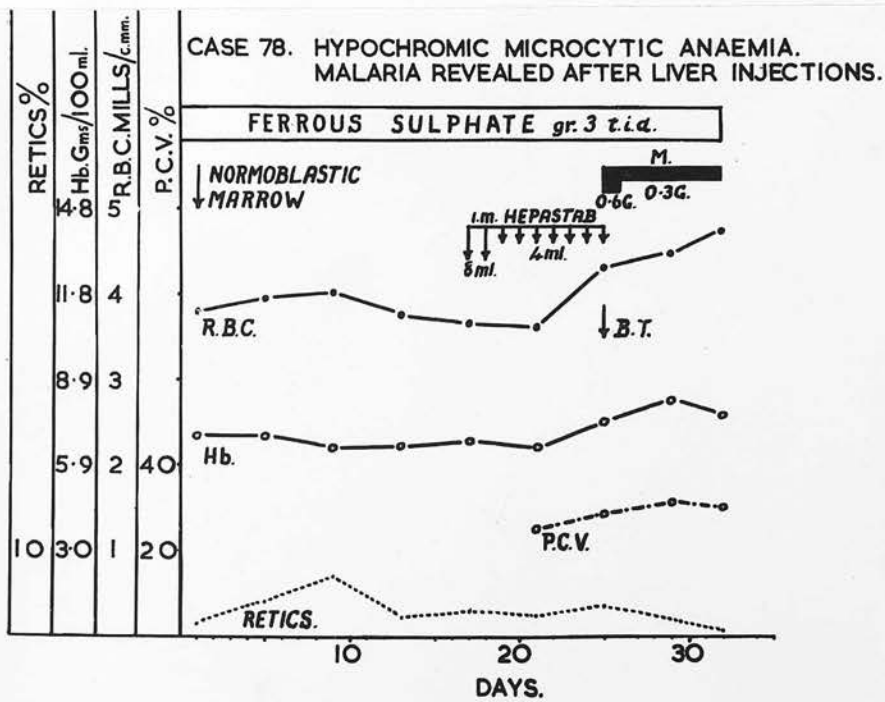
GRAPH 5



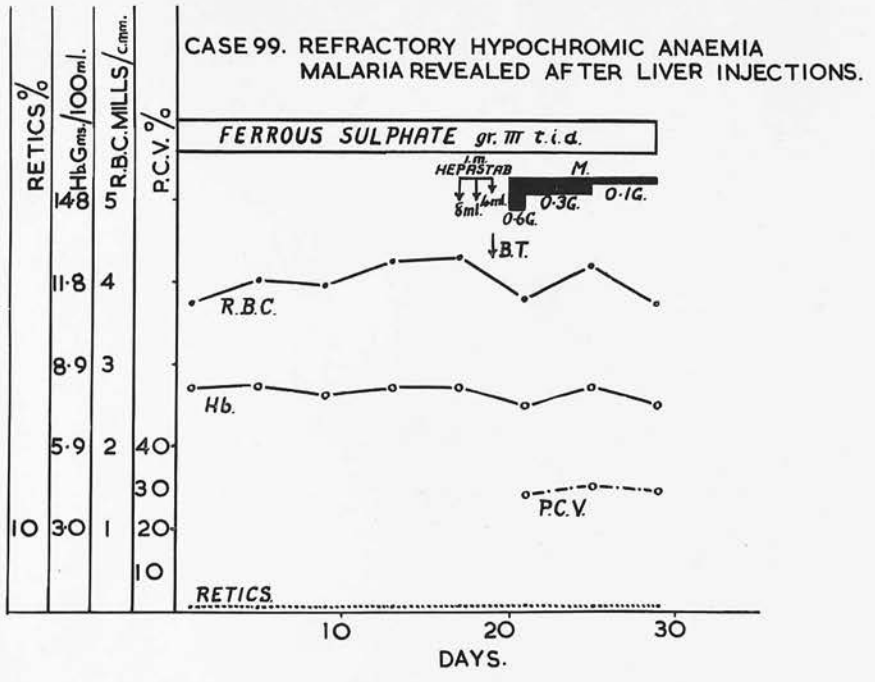
GRAPH 6.



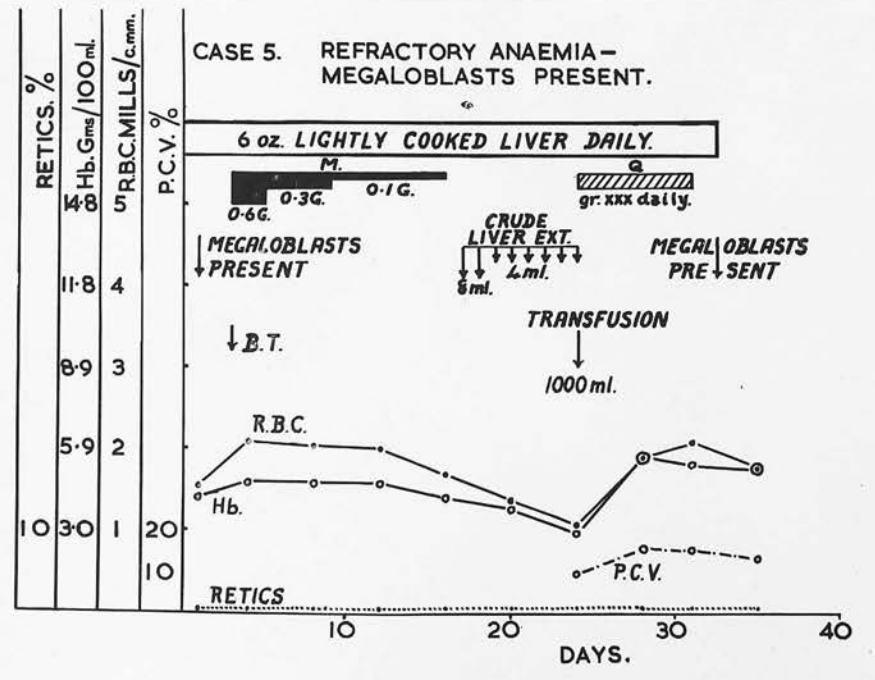
GRAPH 7



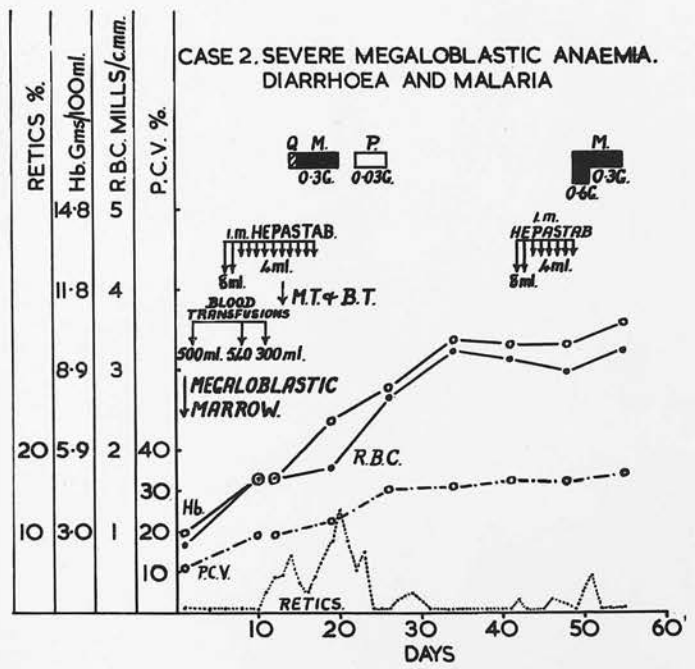
GRAPH 8.



GRAPH 9.



GRAPH 10.


CASE 2. SEVERE MEGALOBLASTIC ANAEMIA.
DIARRHOEA AND MALARIA
RETICS %.
Hb. Gms/100ml.
R.B.C. MILLS/c.mm.
P.C.V. %.
Q M. 0·3G.
P 0·03G.
M. 0·3G.
0·6G.
I.m. HEPASTAB. 4ml. 8ml.
I.m. HEPASTAB 4ml. 8ml.
M.T. & B.T.
BLOOD TRANSFUSIONS
500ml. 500 300ml.
MEGALOBLASTIC MARROW.
R.B.C.
Hb.
P.C.V.
RETICS.
20
10
14·8
11·8
8·9
5·9
3·0
5
4
3
2
1
40
30
20
10
10
20
30
40
50
60
DAYS

GRAPH II.

treatment of this malaria is followed by haematological and clinical improvement, but frequently it is not. The majority of patients with iron deficiency anaemia responded well to iron therapy with or without tetrachlorethylene or similar substances and do not enter into this survey. We have, however, shown one example of a patient with iron deficiency anaemia who did not improve until he was given liver injections, but then he developed clinical evidence of malaria. Another such patient merely developed malaria after liver injections, without having any haematological improvement.

In the jungle warfare the known factors to be considered were:

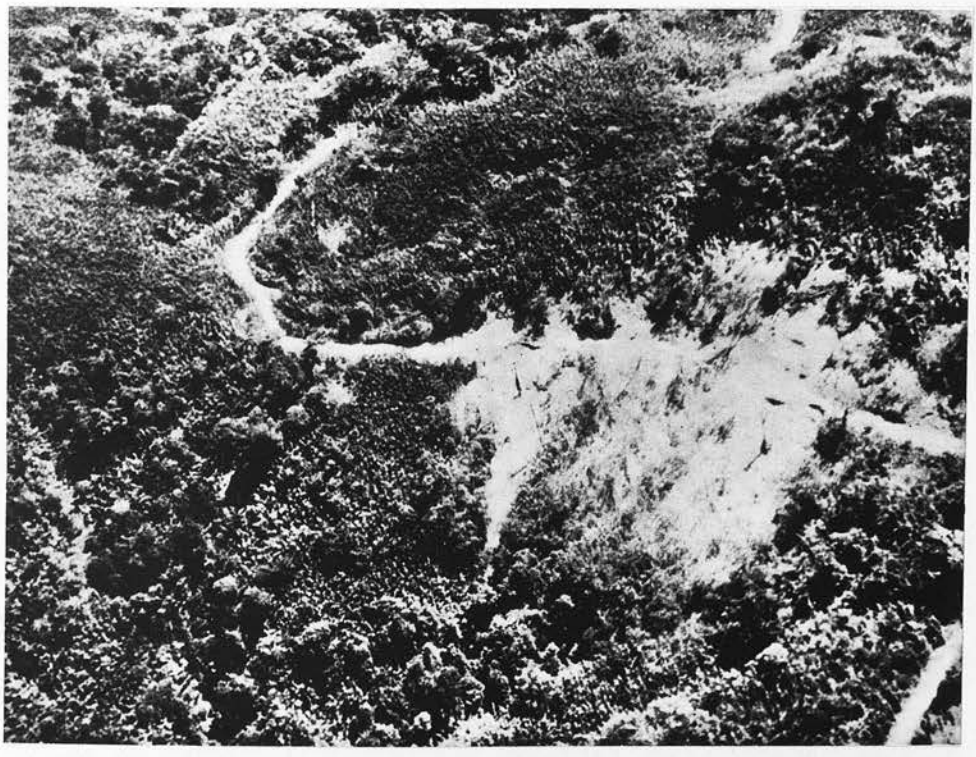
(1) the very high incidence of malarial infection, possibly of a type different to that affecting these men before they crossed the Brahmaputra river. As can be seen from our data, the absence of reticulocytosis, icterus of the serum or splenic enlargement were not reliable indications of the absence of malarial infection.

(2) the fact that the malaria was suppressed by mepacrine.

(3) the increased incidence of sprue amongst troops stationed east of the Brahmaputra river. Leishman (1945) has recorded that in one R.A.F. unit, within three weeks of its arrival in the Chittagong area, 10% of its personnel was ill with diarrhoea which rapidly developed into the full sprue syndrome. It has/

Conditions on the Arakan front.

Parachute dropping of supplies.



Typical terrain in Burma.

has been customary to state that "sprue does not occur in Indians" and partly for this reason Chaudhuri & Chaudhuri (1944), describing a sprue-like condition in Indians in Calcutta, used the name 'parasprue'. In 1944, too, Cook described a sprue-like condition which occurred frequently in Gujerat, Southern India. There seems little reason for using a name other than tropical sprue for the condition affecting these patients. The lower fat content of the stool that has been shown to occur in the Indian with 'parasprue' compared with that obtaining in the European with tropical sprue, may merely be a reflection of the lower fat intake of the Indian diet.

(4) the increased prevalence of dysentery may have been a precipitating factor in previous years, but not in 1944-45.

(5) the poor diet supplied to some units, especially where fighting was heaviest and where supplies had to be dropped by parachute. This had been more of a problem the previous year.

The question of diet was particularly difficult to assess in our cases, but in general these men could not have been faring too badly as regards total caloric intake. It seems likely that their bodily reserves of folic acid and vitamin B_{12} were never high, because of years of deficient intake of these substances and that it is this difference between the/

the British and the Indian soldier that explains the disparity in the incidence of megaloblastic anaemia in British and Indian units fighting side by side. It will be recollected, however, that Hynes did not find macrocytic anaemia to be a problem in any of his surveys of Indian soldiers and all that can be said now is that this possible tissue deficiency was a question that could not be investigated in 1944 but which could probably now be solved by means of the folic acid excretion tests, the serum vitamin B_{12} estimations and the tissue assay techniques that are described later in this thesis.

(6) As has been seen some of the men with megaloblastic anaemia had not taken part in jungle warfare, but it is reasonable to consider the possibility that where arduous fighting and forced marches took place in the jungles, this increased the utilisation of vitamin B_{12} and folic acid in the body.

(7) A further consideration that arises concerns the possibility that changes in the intestinal flora may have played a part in the development of megaloblastic anaemia. This is a factor that is always being quoted, but even now it is difficult to suggest a satisfactory method for the evaluation of this aspect of the pathogenesis of the megaloblastic anaemias. Most of the men said that their food was satisfactory but that the water they drank was not. Apart from the/

the water which was frequently chlorinated, it is likely that suppressive mepacrine altered the intestinal flora. (See also Chapter 5.)

(8) Ankylostomiasis did not seem to be a factor of primary importance in macrocytic anaemia.

When one studies Table 14 and the abstracts of case records that follow it, one cannot help being struck by two things — the poor response to liver therapy, and the high incidence of malaria.

It is unfortunate that these investigations had to be done before the days of microbiological assay of liver extracts for their content of cyanocobalamin or folic acid. (We have recently attempted, unsuccessfully, to obtain ampoules of the batch of Hepastab used in the investigation.) It may be that the autolysed yeast, the "Hepastab", the TCF liver extract and the liquid liver extract for oral administration were all non potent, and that although the Hepastab and TCF extract had been tested clinically in pernicious anaemia this was of no assistance since the real deficiency was in folic acid, not vitamin B_{12}. If this is so we were more unfortunate than other investigators in having no potent remedies, and at least two of our patients did not respond to lightly cooked liver given by mouth. Our efforts to obtain proteolysed liver were unsuccessful because the factory had been bombed.

On the other hand it seems possible that the particulars of Case 44, the British soldier who undoubtedly had refractory macrocytic anaemia due to benign tertian malaria, are instructive. Here there was no complication of primary mal-

nutrition/

malnutrition, no clinical suggestion of sprue and no ankylostomiasis, but the anaemia persisted despite antimalarial treatment, and repeated relapses showed that the malarial infection had not been eradicated. Taliaferro & Mulligan (1937) have referred to the possible phagocytosis of normal red cells by the hypertrophic reticulo-endothelial system in monkey malaria.

In haemolytic anaemia, as will be mentioned again later (p.520), megaloblastic anaemia very rarely occurs, even in well fed persons. If Case 44 had been a relatively poorly fed Indian instead of a well fed European it would not have been surprising to find that he had a refractory megaloblastic anaemia associated with his malaria instead of a refractory macrocytic anaemia with normoblastic marrow. If indeed poor nutrition leads to folic acid deficiency and chronic malaria causes increased demands for haemopoietic factors in the very people who have the poorest stores of these, the surprising fact is not that these men developed megaloblastic anaemia, but that its occurrence in association with malaria is not more widespread. In addition to these two possible effects, it seems that in some of our patients chronic malaria led to fever and anorexia, with resultant diminution in the intake of foodstuffs, thus making matters even worse.

These various possible factors are shown in Fig. 1. (p.129).

NUTRITIONAL MACROCYTIC ANAEMIA IN JAPANESE PRISONER OF WAR CAMPS.

Strangely enough nutritional macrocytic anaemia was not a problem amongst Europeans and Australians in Japanese P-O-W camps./

camps. A comprehensive account of illnesses amongst the unfortunate captives, entitled "Deficiency Diseases in Japanese Prison Camps" has been published in the M.R.C. Special Report Series by Smith & Woodruff. On page 146 of this Report it is stated (in relation to white prisoners) that the only camp in the Far East in which nutritional macrocytic anaemia occurred was the civilian camp at Hong Kong, where there were fifty-five cases. From this cause there were ten deaths, four occurring in 1942, the year of capture, two in 1943, three in 1944 and two in 1945. Eleven of the fifty-five patients so affected were pregnant women and of the ten women known to have become pregnant during the last year of internment, seven developed macrocytic anaemia. The only cases of scurvy that occurred were in this same camp, a fact that may be significant in that green leaves are a source both of folic acid and of ascorbic acid.

When the war in the Far East collapsed, the present author was on his way to Rangoon to commence the second stage of the investigation of anaemia in Indian troops on active service. The plan had to be abandoned, but it was possible instead to carry out a clinical and haematological survey of 106 Europeans and Australians who had just been released from Japanese P-O-W camps in Thailand and immediately evacuated to Rangoon. The seriously ill patients remained in Thailand and the cases were further selected in that these men with severe loss of vision due to malnutrition were all sent to the hospital in which this survey/

survey was carried out. The eyes were examined by Major H. Ridley, R.A.M.C., Ophthalmic Specialist, and the degree of visual loss, where it occurred, is included in Table 16. These men had been in captivity for $3\frac{1}{2}$ years, and many had taken part in the building of the Burma-Thailand railway or the notorious Mergui Road. One patient who was employed on the latter project stated that of the original number of a thousand in his party, approximately four hundred men had died of disease or exhaustion.

The main article of diet was rice, and detailed dietetic information is given in the M.R.C. Report on the subject. The captives had organised their medical services well and had kept records of their illnesses, but, of course, had little or nothing to use for treatment. As will be seen from Table 16, malaria had been very prevalent; nevertheless splenomegaly was rare. In the column headed 'Remarks' an indication is given as to whether or not beriberi, pellagra, or the burning foot syndrome had been present during captivity (see Spillane, 1947). Diarrhoea is only mentioned if it had been continuously present for several months. Most of the men had been affected with scrotal dermatitis, especially in 1943, and it is only indicated here if it had been particularly severe.

On physical examination most of the men were in good general health, and apart from the serious visual disturbance there was little abnormal to be found. It will be seen that, although anaemia was not a marked feature, about half the men had a red cell count of less than 5,000,000 per c.mm., and that the/

TABLE 16.

BLOOD COUNTS AND OTHER FINDINGS IN EUROPEANS AND AUSTRALIANS IMMEDIATELY AFTER RELEASE FROM JAPANESE POW CAMPS IN THAILAND, SEPTEMBER 1945.

Case No.	Hb. G%	RBC mills/ c.mm.	PCV %	MCV cu.	MCHC %	No. of Malarial attacks	Spleen	Vision Rt. Eye	Vision Lt. Eye	Remarks
1	10.1	3.0	27.6	90.2	36.7	1	-	6/60	< 6/60	Emaciated, Pulm. T.B.
2	12.5	3.36	36.0	107.1	34.8	21	-	6/60	2/60	B
3	10.1	3.59	29.0	80.5	34.8	+++	-	6/12	6/60	B
4	12.1	3.64	35.0	96.1	34.5	-	-	6/60	3/60	-
5	11.5	3.86	34.2	88.6	33.5	20	+	6/36	nil	-
6	13.4	3.88	38.0	97.9	35.3	++	-	-	-	-
7	12.4	3.88	35.5	91.4	34.8	2	-	6/60	nil	B,P
8	12.6	3.91	37.0	94.6	34.1	44	-	6/24	6/24	B
9	13.3	3.95	43.0	108.8	30.8	++	-	6/18	6/24	B
10	14.4	4.00	41.0	102.5	35.1	+++	+	6/60	6/5	-
11	14.0	4.00	39.0	97.5	35.9	14	-	-	-	-
12	14.2	4.10	43.0	104.8	33.0	+++	-	1/60	1/60	P
13	13.4	4.10	41.0	100.0	32.7	20	-	6/36	6/60	P Emaciated
14	14.0	4.13	38.5	93.2	36.4	+++	-	6/24	nil	P
15	15.9	4.20	46.9	111.6	33.9	-	-	-	-	-
16	12.2	4.20	35.6	84.7	34.3	30	-	6/24	6/60	-
17	15.3	4.30	41.0	95.3	37.6	40	-	-	-	B
18	14.0	4.31	40.0	92.8	35.0	++	-	-	-	-
19	11.8	4.33	34.0	78.5	34.6	++	-	-	-	B
20	15.6	4.38	47.8	109.1	32.7	+	-	-	-	B
21	13.7	4.40	42.0	95.5	32.6	++	+	-	-	B
22	14.0	4.40	41.5	94.3	33.7	+++	-	2/60	2/60	B,S
23	14.5	4.40	40.0	90.9	36.1	20		6/24	6/24	-
24	14.8	4.41	44.0	99.7	33.5	+	-	6/6	6/6	-
25	14.6	4.44	42.0	94.6	34.8	3	-	-	-	Thin
26	15.2	4.44	44.0	99.9	34.5	+++	-	-	-	B,S
27	14.5	4.45	41.5	93.7	34.8	nil	-	6/60	6/60	B
28	12.7	4.46	34.5	77.3	36.7	14	-	6/60	6/60	-
29	15.6	4.50	45.0	100.0	34.8	8		-	-	B
30	14.2	4.52	39.5	87.6	35.8	22	-	-	-	B
31	13.9	4.52	41.8	92.4	33.1	+	-	6/5	6/5	P
32	13.6	4.55	38.2	86.3	35.6	2	-	6/36	6/60	B,S
33	15.0	4.63	38.8	83.8	38.8	+	+	-	-	Has congenital haemolytic anaemia
34	15.1	4.64	45.5	98.0	33.2	30	-	4/60	5/60	P
35	15.3	4.70	46.0	97.8	33.3	7	-	6/36	6/36	B
36	15.0	4.70	41.0	87.2	36.7	21	-	6/6	6/60	B
37	14.9	4.71	41.5	88.1	35.9	++	-	6/60	6/60	Glossitis present
38	15.0	4.72	43.2	91.5	34.8	++	-	6/60	6/60	B,D (2½ years)
39	14.5	4.72	42.0	88.9	34.4	5	-	6/60	6/60	B,P
40	14.0	4.75	41.0	90.5	34.1	50	-	6/60	H.M.	-
41	15.0	4.75	44.1	92.8	34.1	10	-	6/60	3/60	B
42	14.3	4.76	38.6	81.0	37.1	4	-	6/36	< 6/60	B, Deaf
43	15.3	4.77	45.0	94.3	34.1	-	-	6/60	6/60	B,P
44	15.0	4.82	43.0	89.2	34.9	4	-	-	-	B,P
45	14.6	4.82	42.4	98.1	34.4	-	-	3/60	6/60	-
46	14.9	4.85	40.0	82.4	37.3	-	-	6/60	6/60	B,S
47	16.8	4.85	49.4	101.6	34.1	8	-	6/60	6/36	-
48	16.2	4.87	45.3	96.3	35.7	-	-	-	-	B,P
49	15.6	4.90	44.5	90.8	35.1	-	+	6/9	6/12	-
50	16.7	4.92	46.0	93.4	36.3	+	-	< 6/60	< 6/60	B,P
51	14.5	4.92	41.0	83.3	35.2	12	-	6/36	6/36	D (18 mths.) Thin
52	16.5	4.95	48.0	96.9	34.4	++	-	-	-	B
53	14.8	4.96	42.0	84.6	35.1	75	-	6/60	6/60	F

* B = Beri beri; D = Chronic diarrhoea; F = Burning feet; P = Pellagra; S = Scrotal dermatitis.

TABLE 16.

BLOOD COUNTS AND OTHER FINDINGS IN EUROPEANS AND AUSTRALIANS IMMEDIATELY AFTER RELEASE FROM JAPANESE POW CAMPS IN THAILAND. SEPTEMBER 1945.

Case No.	Hb. G%	RBC mills/ c.mm.	PCV %	MCV cu.	MCHC %	No. of Malarial attacks	Spleen	Vision Rt. Eye	Vision Lt. Eye	Remarks
54	16.4	4.97	43.2	86.9	37.9	33	-	-	-	B
55	15.3	4.99	44.8	89.7	34.2	26	-	-	-	-
56	15.2	5.00	46.0	92.0	33.0	5	-	6/18	6/12	S,P,B,D
57	16.2	5.00	46.0	92.0	35.3	+	-	6/36	6/36	-
58	16.2	5.00	46.0	92.0	35.3	1	-	3/60	3/60	P,B, Paralysed 18 mths.
59	15.6	5.01	41.0	81.8	38.1	10	+	-	-	S
60	15.2	5.01	43.0	85.8	35.4	30	-	6/36	6/36	B,D, ++
61	15.8	5.03	47.4	93.5	33.3	35	-	6/18	6/18	-
62	15.8	5.06	46.5	91.9	33.4	4	-	6/24	6/24	B,P
63	15.9	5.08	42.0	82.4	38.0	+++	-	6/36	6/60	-
64	16.2	5.09	44.0	86.4	37.0	2	-	6/60	6/60	-
65	15.4	5.09	41.5	91.3	37.0	1	-	6/12	6/18	-
66	16.4	5.09	49.0	96.2	33.5	15	-	-	-	-
67	16.0	5.10	46.2	90.5	34.4	1	-	6/5	6/5	B,P,S
68	16.4	5.10	47.0	92.1	34.9	-	-	5/60	6/60	-
69	14.8	5.14	38.1	74.1	38.7	+++	+	-	-	-
70	16.1	5.16	42.5	82.3	37.9	29	-	6/36	6/36	-
71	17.0	5.17	49.8	96.3	34.2	-	-	6/24	6/18	-
72	16.3	5.19	48.0	92.5	34.0	60	-	-	-	B,P,D (9 mths.)
73	15.5	5.20	43.0	82.7	36.0	++	-	-	-	B,D (2½ years)
74	15.3	5.20	43.2	83.0	35.5	3	-	-	-	P
75	15.0	5.20	38.4	73.8	39.2	-	-	-	-	B,P
76	15.9	5.22	47.0	90.0	33.9	35	-	-	-	B,P
77	15.5	5.22	44.0	84.3	35.2	30	-	6/60	6/60	-
78	16.7	5.24	46.2	88.2	36.1	-	-	-	-	-
79	15.3	5.25	44.3	84.3	34.6	30	-	< 6/60	< 6/60	B,P
80	17.1	5.28	44.4	84.0	38.6	1	-	-	-	B
81	17.9	5.30	52.0	98.1	34.4	20	-	-	-	B,P
82	16.1	5.34	47.0	89.0	34.2	1	-	-	-	-
83	15.9	5.34	44.3	82.9	36.0	++	+	-	-	B,D
84	17.7	5.36	47.6	88.8	37.2	+	-	-	-	-
85	15.3	5.38	42.0	78.0	36.5	2	-	6/60	6/24	S,B
86	15.5	5.41	42.0	77.6	36.9	1	-	-	-	Thin
87	15.6	5.42	44.6	82.3	32.8	-	-	6/36	6/36	S
88	16.3	5.43	49.5	92.6	35.0	47	-	6/36	6/36	B,P,S,D
89	16.8	5.45	48.2	88.2	34.9	27	-	6/36	6/18	B
90	16.4	5.45	48.2	88.7	34.9	-	+	-	-	F
91	15.0	5.47	44.8	81.9	33.6	30	-	6/18	6/18	Deaf
92	16.7	5.48	45.8	83.6	36.4	2	-	6/36	6/60	Deaf
93	16.7	5.57	46.0	80.7	36.3	25	+	3/60	3/60	Deaf
94	14.9	5.57	43.0	77.2	34.7	+++	-	6/60	6/60	B
95	17.7	5.59	48.4	86.5	36.6	+	-	-	-	-
96	16.7	5.68	48.7	84.4	34.3	+	-	6/18	6/9	P
97	18.0	5.69	48.5	85.2	37.1	30	-	-	-	-
98	16.0	5.70	46.2	81.0	34.8	10	-	6/36	6/6	B
99	16.2	5.76	48.1	83.5	33.7	-	-	-	-	B
100	16.8	5.77	44.6	79.0	37.7	9	-	6/18	6/18	B,S
101	16.2	5.80	45.0	77.5	36.0	27	+	-	-	B
102	16.0	5.83	45.9	78.7	34.9	+++	+	-	-	S
103	17.0	5.85	48.0	82.5	35.4	-	-	6/24	6/36	B
104	16.4	5.88	44.0	73.1	37.3	38	-	6/36	6/36	B,P,D
105	18.6	5.90	50.8	88.8	36.6	12	-	-	-	-
106	18.6	5.90	49.0	83.0	38.0	1	-	6/9	6/9	B

* B = Beri beri; D = Chronic diarrhoea; F = Burning feet; P = Pellagra; S = Scrotal dermatitis.

the anaemia, where present, tended to be macrocytic. The blood films appeared normal apart from slight macrocytosis and anisocytosis where there was definite anaemia: the carrying out of sternal puncture was, rightly, strictly forbidden.

Although it is true that there had been an improvement in the food supply in the four months before the men were released it is perhaps surprising that, for example, a man like Patient 88 of Table 16, who had had beri beri, pellagra, severe scrotal dermatitis, continuous diarrhoea for several months and forty-seven attacks of malaria should, despite the continuation of a rice diet, have a haemoglobin level of 16.3 G. per 100 ml., with a red cell count of 5,430,000 per c.mm. and a haematocrit reading of 49.5%. The infrequency of splenomegaly is also to be noted.

Since there could be no direct measurements of the content of folic acid and vitamin B_{12} in the diets of the men in those P-o-W camps, it would serve little purpose to attempt to give accurate figures for these now. The fact is that nutritional megaloblastic anaemia was not a feature and therefore it must be taken that the body stores of folic acid and vitamin B_{12} were sufficient in the European and Australian prisoners, together with what was supplied in the diet, to prevent megaloblastic anaemia in the vast majority for three and a half years. Our knowledge of the time that it takes for megaloblastic anaemia to develop in an otherwise healthy person deprived of vitamin B_{12} in the diet is sketchy, since the pernicious anaemia patient under treatment is not a normal subject for studies of this nature/

nature. (See also p.271).

Little has been published about the condition of Indian troops released from Japanese P-O-W camps, but Walters et al. (1946) report that the great majority of these men had anaemia with a high MCV and normal MCHC. The mean haemoglobin reading on 27 of their representative patients was 10.5 G. per 100 ml.

In May 1945 the present author carried out a haemoglobin survey on 225 Indian prisoners who had deserted to or been captured by the Japanese and had been recaptured later while fighting against the British. These men had minor ailments or minor surgical conditions and were in the course of evacuation to bases in India. There was no beri beri, pellagra, ariboflavinosis, scrotal dermatitis or optic atrophy, but the mean haemoglobin level was only 12.65 G. per 100 ml.

The main possible relevant differences between the conditions of our Indian troops who did develop megaloblastic anaemia readily, and the released European and Australian prisoners who did not were:-

(1) The prisoners were fed highly milled rice, whereas the Indian troops were supposed to be supplied with undermilled or parboiled rice. Unless there are antimetabolites of folic acid or vitamin B_{12} in the husks of rice, this should have been to the advantage of the Indians.

(2) The prisoners received about half the number of calories that the Indians were supposed to be having. It is generally considered that the necessary intake of vitamins depends upon the total caloric intake, and although the Indian diet was not always up to the ration scale, this deficiency did not continue for periods of years as was the case in the prison camps.

(3) The prisoners made every effort to supplement their diet with such things as green vegetables, grass extracts, grass polishings, various roots, gram and rice polishings. The Indian/

Indian troops, in general, took what was given to them.

The Indian basic ration scale provided 75 mg. of ascorbic acid daily and it is certain that this level was frequently not reached on active service except by the provision of multivitamin tablets. The prison camp diets provided as much or more ascorbic acid, and this is calculated by Smith & Woodruff from the actual diets consumed. Since a main source of ascorbic acid was green leafy vegetables and as these have a good content of folic acid, this is indirect evidence of the presence of a reasonable amount of folic acid in the prisoners' diet.

(4) The animal protein content of the prisoners' diet was lower than that in the Indian ration scale, but then many Indians were vegetarians and many non-vegetarians did not receive the authorised scale of rations.

(5) Sprue did not occur in the P-o-W camps, possibly in part because of the low fat intake. Where it was present, the patients became seriously ill or died on their release and return to normal diet. (Price, 1946).

(6) Malaria was very prevalent amongst the prisoners and was not adequately treated. Our Indian troops took suppressive mepacrine, but megaloblastic anaemia east of the Brahmaputra river was a problem before this was introduced. Splenomegaly was strangely uncommon amongst the prisoners, and it is tempting to suggest that anaemia due to hypersplenism was a factor in the Indians and not in the prisoners. It is well recognised that within the four species of Plasmodium parasitizing man there appear to be many strains which are morphologically alike but different immunologically and in other ways. This may be of importance in relation to the difference in the degree of splenic enlargement in the two groups of cases and also in relation to the development of a refractory form of anaemia particularly in Assam and certain areas of Burma.

(7) There may have been differences in the intestinal flora from variations in diet and water supply.

(8) It is difficult to escape the conclusion that the basic difference was that the Indian soldier went into the jungle fighting areas with low bodily reserves of many things including folic acid and vitamin B_{12}, while the white prisoners entered the prison compounds with normal tissue stores.

General Conclusions.

Tropical/

Tropical macrocytic anaemia is not a disorder or syndrome, but a complex series of disorders. The possible causes are many and these vary not only from country to country but also in different areas of the same country.

Although it has not been possible in the past to make measurements of the folic acid or vitamin B_{12} content of the body tissues and organs, the indirect evidence suggests that the Indian is fundamentally prone to megaloblastic anaemia because of years of under-nutrition and consequent poor bodily stores of antimegaloblastic substances. It is hoped that the new biochemical tests to be described later in this thesis will in the course of time, give an absolute answer to this aspect of the problem of megaloblastic anaemia in the tropics.

FIG. I. FACTORS THAT MIGHT CAUSE MEGALOBLASTIC ANAEMIA IN INDIAN TROOPS ON THE EASTERN FRONT.

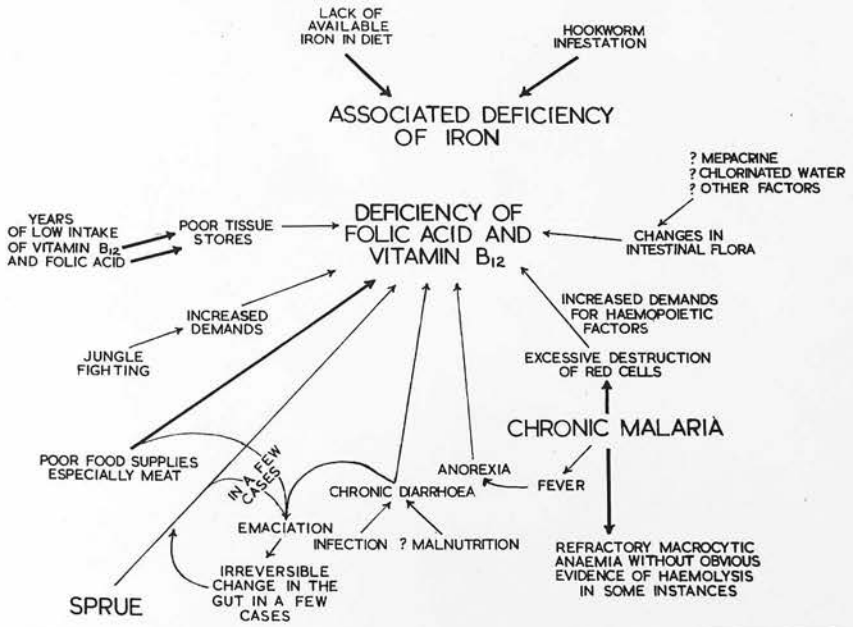


FIG.2. FACTORS THAT APPEAR TO HAVE BEEN OF IMPORTANCE IN CAUSING MEGALOBLASTIC ANAEMIA IN WILLS' SERIES

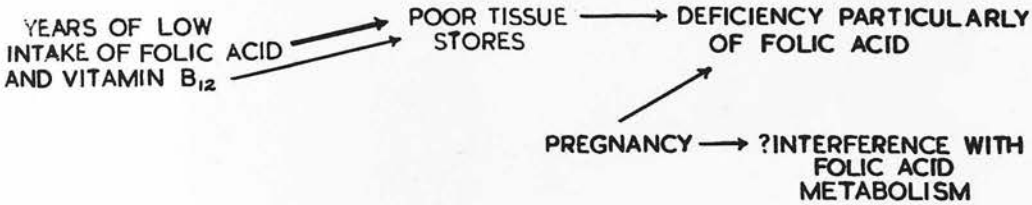


FIG.3. FACTORS THAT APPEAR TO HAVE BEEN OF IMPORTANCE IN CAUSING MEGALOBLASTIC ANAEMIA IN NAPIER'S SERIES & DAS GUPTA'S SERIES.

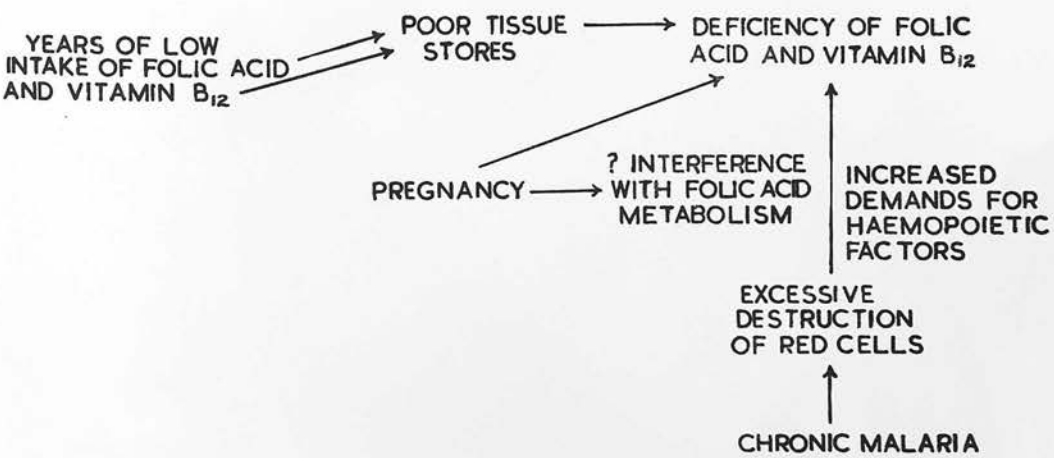


FIG. 4. FACTORS THAT APPEAR TO HAVE BEEN OF IMPORTANCE IN CAUSING MEGALOBLASTIC ANAEMIA IN FAIRLEY'S SERIES IN MACEDONIA

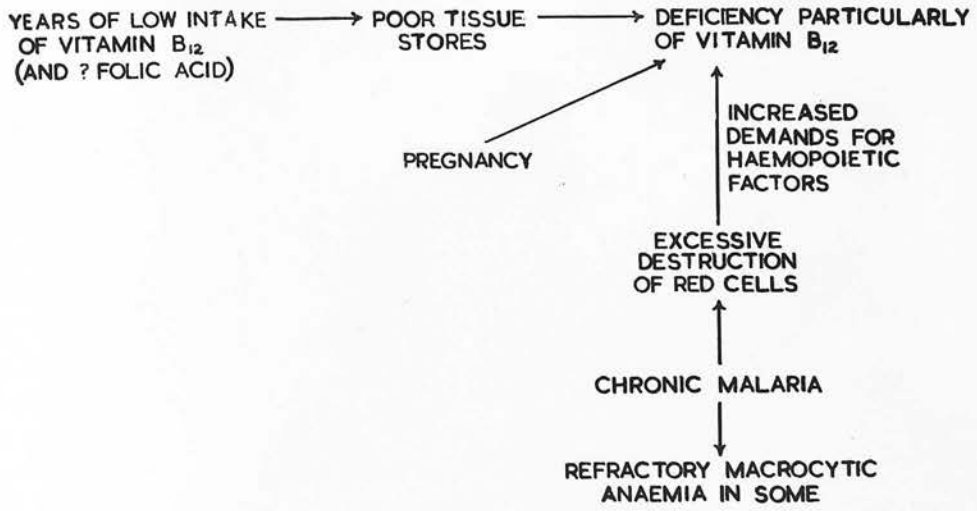
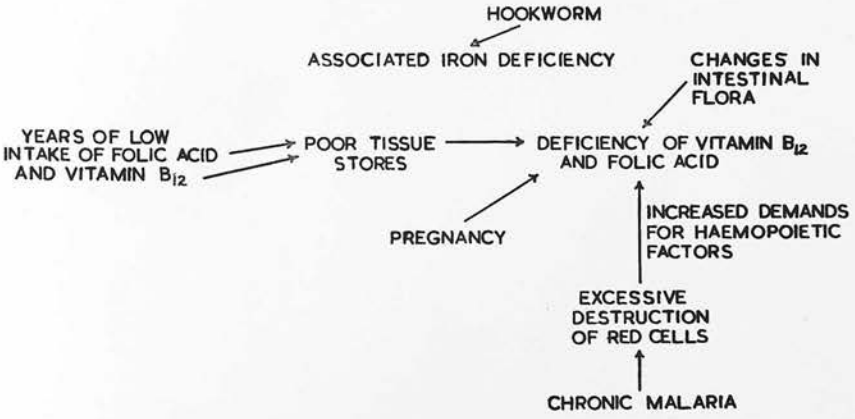


FIG. 5. * FACTORS THAT APPEAR TO BE OF IMPORTANCE IN CAUSING MACROCYTIC & MEGALOBLASTIC ANAEMIA IN FOY & KONDI'S CASES IN AFRICA.



* NOTE. TROWELL'S CASES MIGHT BE EXPECTED TO BE CAUSED BY SIMILAR FACTORS. THE ABSENCE OF MEGALOBLASTOSIS IN THEM IS MOST SURPRISING, AS IS THE THEORY THAT THE MACROCYTOSIS IN THEM IS DUE TO RETICULOCYTES AND OTHER YOUNG CELLS

CHAPTER 3.

THE PRODUCTION OF MEGALOBLASTIC ANAEMIA IN ANIMALS WITH AN ACCOUNT OF EXPERIMENTS DESIGNED TO PRODUCE MEGALOBLASTIC ANAEMIA IN THE GUINEA PIG

REVIEW OF LITERATURE

Attempts to produce megaloblastic anaemia in animals have been made by feeding diets deficient in folic acid or vitamin B_{12}, by giving highly purified diets supplemented with sulphathiazole, succinylsulphathiazole or antibiotics, by administering folic acid antagonists, by a combination of these methods, and by performing gastric or intestinal operations.

DIETETIC EXPERIMENTS.

Miller & Rhoads (1933, 1935) reported the production of a megaloblastic anaemia in dogs and pigs fed a purified diet, but their description does not make it clear whether the marrow was truly megaloblastic and, as has been pointed out by Cartwright et al. (1949), the anaemia in the swine was not macrocytic. The dogs developed a syndrome similar to sprue in man. Wills & Stewart (1935) and Wills, Clutterbuck & Evans (1937) produced a megaloblastic anaemia in monkeys by using a diet that was probably deficient in folic acid (Thiersch & Philips, 1949). This did not respond to the parenteral administration of a factor in Campolon that was insoluble in saturated ammonium sulphate and corresponded to Anahaemin, but did respond to the fraction/

fraction that was soluble in ammonium sulphate.

In 1935, too, Day and his co-workers produced 'nutritional cytopenia' and anaemia with diarrhoea in monkeys by means of a diet of polished rice, ground wheat, purified casein, cod liver oil, salt mixture and orange. The syndrome could be prevented by an unidentified 'vitamin M' present in yeast and liver. It was later shown that 'vitamin M' had the properties of folic acid. In 1951, Smith & Elvehjem produced in young rhesus monkeys a syndrome of anaemia, granulocytopenia, impaired growth and dysentery by dietetic means. Marrow punctures were not performed. The syndrome could be abolished by daily oral doses of 1 mg. pteroylglutamic acid or by a 60 per cent methanol extract of liver. It was suggested that the pteroylglutamic acid might be acting indirectly by stimulating the biosynthesis of an unknown monkey antianaemic factor (MAAF). A 60 per cent methanol extract of liver is a good source of MAAF, and this factor differs from cyanocobalamin, pteroylglutamic acid, citrovorum factor or ascorbic acid. There appeare to be some complex metabolic interrelationship between these factors in the animals, but the relationship is not yet clear. The main point that emerges is that yet another antianaemic factor seems to be present in liver, since the effectiveness of the methanol extract could not be explained by its content of any known antimegaloblastic substance.

It should perhaps be stated here that in the monkey a deficiency of pteroylglutamic acid does not lead to megaloblastic anaemia unless there is at the same time a deficiency of ascorbic/

ascorbic acid. (May et al., 1950).

Ruegamer et al. (1948) have shown that if young growing dogs are fed a purified black tongue-producing diet containing one per cent succinylsulphathiazole supplemented with nicotinic acid and folic acid, diarrhoea, anaemia and a flaccid type of paralysis persist, but complete remission of the anaemia occurs when small quantities of purified liver extract are given in addition. Cartwright et al. (1946) and Cartwright & Wintrobe (1949) produced by dietetic means a normocytic anaemia in pigs that responded to purified liver extracts given intramuscularly, but Welch et al. (1947) failed to produce anaemia with a similar diet. It is possible to produce, by dietetic means, impaired growth in baby pigs and Johnson et al. (1950) have shown that pteroylglutamic acid is required by the pig even in the presence of an adequate supply of vitamin B_{12} and that vitamin B_{12} is required even in the presence of excess pteroylglutamic acid.

The Use of Folic Acid Antagonists. The term folic acid antagonists is applied to certain synthetic analogues and derivatives of pteroylglutamic acid that have the property of inhibiting the growth of the test organisms Streptococcus faecalis R and Lactobacillus casei in the presence of marginal levels of pteroylglutamic acid, the inhibition being reversed by a rise in the concentration of PGA in the culture medium. These folic acid antagonists may produce a state of folic acid deficiency in animals and man, but may differ in this respect in various species.

The/

The groups of workers in Cleveland, Ohio and in Salt Lake City, Utah referred to above have been particularly interested in the production of megaloblastic anaemia in the pig. The earlier experiments concerned the use of a highly purified diet essentially free of vitamin B_{12} and containing sulphasuxidine and a crude x-methyl folic acid antagonist prepared by allowing 2,4,5-triamino-6-hydroxypyrimidine and p-aminobenzoyl-l(+)-glutamic acid to react with 2,3-dibromobutyraldehyde. This treatment, which was at the time considered to be capable of producing a deficiency both of vitamin B_{12} and of folic acid led to the publication of a number of papers (Welch, 1947; Welch et al., 1947, 1948; Heinle et al., 1947, 1948; Cartwright et al., 1948, 1949, 1950). Unfortunately the two groups of workers did not always obtain similar results, and in a later paper Cartwright et al. (1951) stated that the diet they had been using contained 1.1 mμg. of cyanocobalamin per G. of whole diet because the protein of the food had been obtained from an animal source. The earlier experiments of both groups of workers therefore were such as to produce a folic acid deficiency rather than a combined folic acid and vitamin B_{12} deficiency.

Both groups of workers claimed to have produced megaloblastic anaemia in their pigs by the above means. When in the United States the present author made a journey to Cleveland in order to see the marrow films of these animals, but unfortunately Dr. Heinle was unable to find any of them at the time. Both he and Cartwright stated then that the haematological terminology they were using was similar to the one used in Great/

Great Britain (Israels' terminology) and that they had produced cells similar to the intermediate megaloblast of pernicious anaemia. In their 1951 paper, however, Cartwright et al. state that the cells produced in the above experiments were macronormoblasts rather than true megaloblasts.

In any event the macrocytic anaemia produced by the above method did not respond satisfactorily to cyanocobalamin injections, but did respond to pteroylglutamic acid, pteroyldiglutamic acid, pteroyltriglutamic acid and pteroylhexaglutamylglutamic acid. There was a slight and definitely suboptimal haemopoietic response following the administration of crystalline cyanocobalamin, proteolysed liver, marmite, crude desoxyribonucleic acid and crude ribonucleic acid. There was no response to thymine in doses of 10 G. daily, but Welch & Heinle claim a response to thymine in a similar dosage.

Cartwright and his co-workers consider that this porcine anaemia is more like the pteroylglutamic acid responsive liver refractory megaloblastic anaemias (megaloblastic anaemia of pregnancy and of infancy, refractory megaloblastic anaemia) than true pernicious anaemia. It is of interest that, in these pigs, proteolysed liver was more active than marmite although this latter autolysed yeast preparation was found by microbiological assay to contain seven to eight times more folic acid than did proteolysed liver. This may indicate the presence in proteolysed liver of another as yet undiscovered haemopoietic factor.

In one of the Cleveland papers (Heinle et al., 1948) there were described pigs that appeared to become so depleted of vitamin/

vitamin B_{12} and folic acid that eventually folic acid failed to give a response until vitamin B_{12} injections (refined liver injections) were given and, conversely, liver injections failed unless folic acid was given. The general conclusion from these experiments was that the pig had to be supplied with both vitamin B_{12} and folic acid if megaloblastic anaemia was to be prevented.

In their 1951 paper, Cartwright and his colleagues studied 70 pigs. The aim was to produce deficiency of vitamin B_{12} and to this end the purified diet contained soya bean alpha protein in place of casein. Succinylsulphathiazole was added to the diet. As an additional measure to produce vitamin B_{12} deficiency, the operation of gastrectomy was carried out on one group of pigs.

The results of these experiments were that anaemia did not develop consistently, it was never macrocytic when it did develop, the bone marrow appeared normal, and the haematological alterations were neither consistently nor completely corrected by the administration of cyanocobalamin. These results could be explained in two possible ways - either the deficiency was not sufficiently severe to produce megaloblastic anaemia, or there was enough pteroylglutamic acid as a contaminant in the diet to prevent the development of megaloblastic anaemia even in the absence of vitamin B_{12}.

Accordingly yet another series of experiments was carried out/

out (Cartwright et al., 1952). A total of 20 swine were fed a diet adequate in all known respects except that soya bean protein was substituted for casein, succinylsulphathiazole and a folic acid antagonist were added, and cyanocobalamin and pteroylglutamic acid were withheld from the vitamin supplement.

This time the animals developed macrocytic anaemia, leucopenia and neutropenia with erythroid hyperplasia of the bone marrow. The erythroblasts were mainly immature macronormoblasts, but a few atypical megaloblasts were also observed. The anaemia responded rapidly and completely to the administration of pteroylglutamic acid and cyanocobalamin together. The administration of pteroylglutamic acid alone resulted in an immediate return to normality of the blood and bone marrow pictures, but after several months there was a partial relapse in the blood levels despite continued administration of the substance. A response to cyanocobalamin then occurred. The giving of cyanocobalamin alone resulted in only partial remission of the anaemia and the bone marrow remained macronormoblastic although the megaloblasts tended to disappear.

Crude x-methyl folic acid has been used to produce growth defect and normoblastic anaemia in rats (Franklin et al., 1947a), mice (Franklin et al., 1947b; Weir et al., 1948), and impaired growth and anaemia in chicks (Franklin et al., 1947b). These effects could be reversed by folic acid.

4-amino-pteroylglutamic acid (4-amino-PGA: Aminopterin) was found to be highly effective as a folic acid antagonist for bacteria, and has since been tried in mice (Franklin et al., 1948; Philips & Thiersch, 1949), rats (Philips & Thiersch, 1949;/

1949; Oleson et al., 1948; Swendseid et al., 1948) and chicks (Oleson et al., 1948). In these animals the antagonist was very potent, and its effects could not be satisfactorily reversed with pteroylglutamic acid even in relatively large amounts. True megaloblastic anaemia was not produced. Philips & Thiersch (1949) were unable to reverse the effects of 4-amino-PGA with liver extract or thymine in the dosage used, but had some success with pteroylglutamic acid and pteroyltriglutamic acid. The syndrome produced was similar to that of folic acid deficiency.

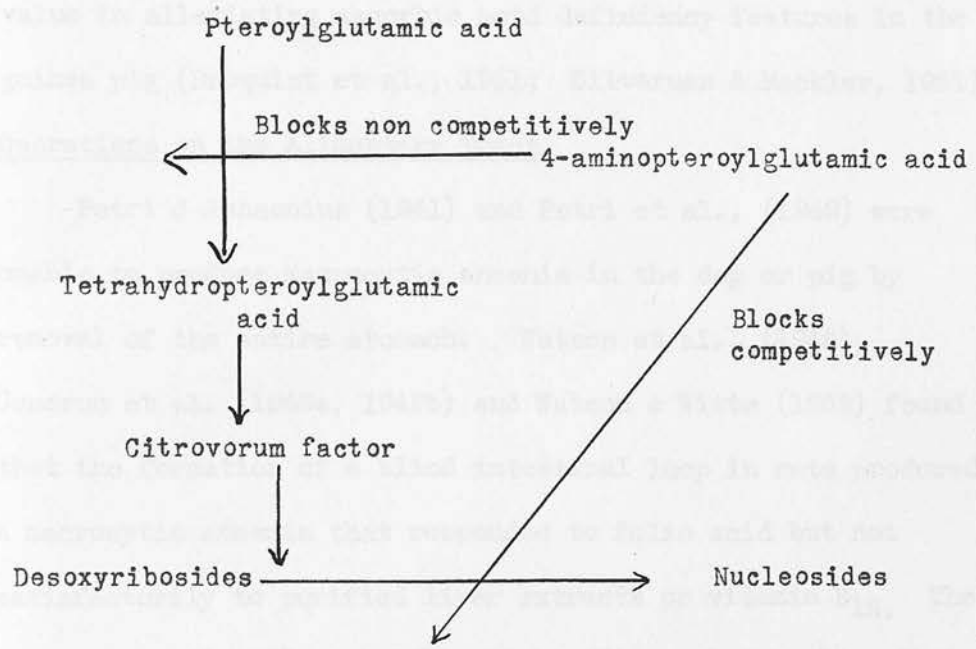
Thiersch & Philips (1949) administered 4-amino-PGA to a series of dogs and produced a sprue-like syndrome with diarrhoea, leucopenia, and a megaloblastic bone marrow. The present author saw colour photographs of these marrow cells and certainly they looked very like the intermediate megaloblasts of pernicious anaemia.

Role of Citrovorum Factor.

In recent years it has been shown that citrovorum factor is much more effective than pteroylglutamic acid in reversing the effects of 4-amino-PGA in experiments with bacteria and animals and in the treatment of malignancy in man. This has been shown in rats (Nichol & Welch, 1950) in mice (Greenspan et al., 1951; Burchenal & Babcock, 1951) and in chicks (Broquist et al., 1951). It is believed that analogues of pteroylglutamic acid with an amino group replacing the hydroxyl group in position 4 (e.g. 4-amino-PGA) have two or more closely related and important sites of action (Welch & Nichol, 1952). First they block profoundly the enzymatic conversion of

pteroylglutamic/

pteroylglutamic acid to citrovorum factor, a step which is believed to be an essential one in the metabolism of pteroylglutamic acid in the body. Secondly it has been postulated that they inactivate an enzymatic component of a system involved in the utilisation of citrovorum factor or block its utilisation in some other way. It is suggested further that this first effect of the 4-amino analogue is exerted in an essentially non-competitive manner, whereas the second effect may be prevented if the enzyme is protected by the administration of sufficient amounts of citrovorum factor to prevent competitively the enzyme from reacting with the analogue.



Role of Ascorbic Acid.

It has been shown by May and his colleagues (1951) that megaloblastic anaemia can be produced regularly in monkeys by the administration of milk diets low in both folic acid and ascorbic acid. Small doses of citrovorum factor were more effective than small doses of pteroylglutamic acid in correcting the anaemia in those monkeys and the investigators concluded that ascorbic acid may be needed for the metabolic production of citrovorum factor. However, ascorbic acid itself caused reversion of the marrow to the normoblastic state. The marrow changes are considered in detail by Sundberg, Schaar and May, 1952.

Neither pteroylglutamic acid nor citrovorum factor is of value in alleviating ascorbic acid deficiency features in the guinea pig (Broquist et al., 1951; Silverman & Mackler, 1951).

Operations on the Alimentary Tract.

Petri & Jensenius (1941) and Petri et al., (1949) were unable to produce macrocytic anaemia in the dog or pig by removal of the entire stomach. Watson et al., (1948), Cameron et al. (1949a, 1949b) and Watson & Witts (1952) found that the formation of a blind intestinal loop in rats produced a macrocytic anaemia that responded to folic acid but not satisfactorily to purified liver extracts or vitamin B_{12}. The marrow was not truly megaloblastic. It was later shown that the anaemia was haemolytic. (Witts, 1953, Personal communication). Reference has already been made to the unsuccessful efforts by Cartwright/

Cartwright et al. (1951) to produce megaloblastic anaemia in pigs by gastrectomy combined with a diet free of vitamin B_{12} and containing succinylsulphathiazole. At the time of the experiments on guinea pigs that will now be described there were no published reports on attempts to produce megaloblastic anaemia in these animals, other than a brief note by Minnich & Moore (1948) who found that 4-amino-PGA in a suitably high dosage gave a hypoplastic anaemia with leucopenia. After the present experiments were completed, however, Innes et al. (1949) reported on the use of the antagonists pteroylaspartic acid, N^{10}-methyl pteroic acid and 4-amino-pteroylglutamic acid. This last was most active, but megaloblastic anaemia did not develop.

ATTEMPTS TO PRODUCE MEGALOBLASTIC ANAEMIA IN THE GUINEA PIG.

Although it is now five years since these investigations were carried out, there has been no other work of a similar nature on the guinea pig. An account of the investigations is included in this thesis because the results are of interest from three points of view:

(a) The anaemia produced in these animals was of a different type to that produced by other investigators using different methods.

(b) The intestinal changes of sprue were not produced. Other workers have suggested that such changes may be produced by 4-amino-pteroylglutamic acid in the experimental animal.

(c) The experimental results require consideration in relation to the possible synthesis of folic acid by organisms in the intestinal tract.

EXPERIMENTAL.

This work was carried out at the Thomas Henry Simpson Memorial Institute, University of Michigan, Ann Arbor, Michigan during the period of tenure of a Rockefeller Travelling Research Fellowship.

Methods Employed.

Various tests were carried out on groups of at least five male guinea pigs of about equal weight. Each test was controlled by the use of a group of untreated animals. All animals, including the controls, received 20 mg. ascorbic acid daily in solution, given by intraperitoneal injection. When 4-amino-pteroylglutamic acid was administered it was given by intraperitoneal/

intraperitoneal injection. All other injections were given intraperitoneally, with the exception of injections of p-amino-benzoic acid which were given into the subcutaneous tissues at the back of the neck. The animals were kept in separate cages with wire-grid bottoms, and were given unlimited amounts of a commercial diet containing a negligible quantity of folic acid, as determined by microbiological assay with S.faecalis as test organism.

Haematological observations.

Cardiac puncture was used to obtain 0.3 ml. of blood, which was then placed in a small tube containing a measured amount of a dry mixture of ammonium and potassium oxalate. White blood cell counts and red blood cell counts were done in duplicate, standardised pipettes being used. Haemoglobin estimations were carried out in a Klett-Summerson photoelectric instrument. Haematocrit determinations were made in Van Allen tubes, which had been centrifuged at 3,000 r.p.m. for 30 min. Peripheral blood examinations were carried out on cover slip films that had been stained with Wright's stain. Differential counts were made on 100 cells. No bone marrow studies were carried out on living animals, but when the animal died or had been killed with ether, cover slip films of the marrow were made and stained with Wright's stain. The organs of all these animals were examined histologically both by Professor C. V. Weller, Professor of Pathology at the University of Michigan and by the present author.

RESULTS

The Production of non-megaloblastic anaemia with 4-aminopteroylglutamic acid. Failure of attempts to/

RESULTS

The Production of non-megaloblastic Anaemia with 4-aminopteroylglutamic Acid. Failure of Attempts to prevent or reverse its Action by Administration of Folic Acid, Liver, Thymine or p-aminobenzoic Acid.

Effects of 4-aminopteroylglutamic Acid alone.

Fifteen animals of about 550 G. weight received 0.25 mg. 4-aminopteroylglutamic acid daily for 4 weeks. Anaemia was evident at the end of the first week, and after 4 weeks the mean red cell count had fallen from 5,320,000 to 4,070,000/cu. mm. The haemoglobin level fell from 14.1 to 11.1 G./100 ml., and the mean corpuscular volume rose from 84.6 to 92.2 cμ. Leucopenia and thrombocytopenia did not develop. A low, continued reticulocytosis was seen (usually 2-7%); this commenced about the third week and was accompanied by the appearance of a few normoblasts in the peripheral blood.

When these animals were killed, the bone marrow was found to be cellular. It contained an increase of haemocytoblasts, proerythroblasts and early normoblasts, but no cells that appeared to be comparable to the early, intermediate or late megaloblasts in man. As in experiments later described by Innes, Innes & Moore (1949), an increase of reticulum cells was seen, and myelocytes were numerous. (Table 19, p.158 and photograph, p.149.) The general condition of the animals remained good, and no marked naked eye or microscopic changes were found in the viscera, apart from some fatty infiltration of the liver.

A further five animals of 431 G. mean weight were given 0.25 mg. 4-aminopteroylglutamic acid daily for 7 weeks. The anaemia continued and, although it varied in degree from week

to week, the mean red cell count at 7 weeks was 4,280,000/cu. mm. and at no time was it less than 3,900,000/cu.mm. (See Table 17.)

Effects of Various Supplements.

Six groups of guinea-pigs of about 550 G. weight received 0.25 mg. 4-aminopteroylglutamic acid daily with the following supplements.

Pteroylglutamic acid. Ten animals were given daily intraperitoneally 2.5 mg., and five 5.0 mg. pteroylglutamic acid.

Thymine. Five animals received approximately 0.45 G. thymine daily, some of it by mouth, some by means of a stomach tube, and the rest of it as an addition to the food.

p-Aminobenzoic acid. Five animals were given daily subcutaneously 2 ml. of a 10% solution of a mixed sodium and potassium salt of p-aminobenzoic acid and had the same salt added as a 2% supplement to the diet.

Liver injections. Five animals received daily intraperitoneally 4 U.S.P. units of a crude liver extract.

Liver by mouth. Five animals had a liver preparation in powder form added to the diet in the proportion of 5%.

As compared with guinea-pigs receiving 4-aminopteroylglutamic acid alone, none of these groups of animals showed any significant alteration in the extent of the anaemia, in the bone marrow changes or in general condition.

Effects of increasing the Amounts of 4-Aminopteroylglutamic Acid and of Pteroylglutamic Acid.

Five animals of 518 G. mean weight were given daily 0.5 mg. 4-aminopteroylglutamic acid and also 5 mg. pteroylglutamic acid intraperitoneally. These guinea-pigs became more severely anaemic, the mean red cell fount falling at the end of the fifth week from 5,410,000 to 2,770,000/cu.mm. and the haemoglobin/

TABLE 17.

Effect of Administration of 0.25 mg. of 4-Amino-pteroyl-glutamic Acid daily to a group of five Guinea pigs.

Time (wks)	Mean Weight (G.)	Hb. G./ 100 ml.	R.B.C. mills/ c.mm.	W.B.C. per c.mm.	Polys. per c.mm.	P.C.V. (%)	M.C.V. (cμ)
	4-amino-pteroylglutamic acid given alone.						
0	431	13.0	5.04	10,160	6070	46.3	92.2
1	452	11.9	4.51	7,310	4000	40.6	90.1
2	475	11.6	4.18	6,250	3800	40.8	97.8
3	511	11.1	4.09	6,630	3370	38.6	99.4
4	526	11.0	4.32	5,920	2850	39.2	91.7
5	550	12.2	4.73	7,800	2870	40.3	85.8
6	575	10.7	3.93	5,240	1900	35.7	91.0
7	582	10.4	4.28	5,280	2000	36.2	85.2

haemoglobin from 13.2 to 7.6 G./100 ml. The animals became lethargic and their hair roughened, but growth continued, although less satisfactorily than in the controls. The polymorphonuclear leucocyte count fell from 4,950 to 630/cu.mm. at the end of the first week, but rose again to 1,250/cu.mm. at the end of five weeks. Similar results were obtained in five animals when 0.5 mg. 4-aminopteroylglutamic acid was given without pteroylglutamic acid. The marrows were similar to those of the animals in Table 17, although the cellularity was perhaps reduced in some.

Five animals that received daily 50 mg. pteroylglutamic acid together with 0.5 mg. 4-aminopteroylglutamic acid, however, became very ill. They rapidly became emaciated, lethargic and dehydrated. Four of the animals died, at 9, 11, 14 and 21 days, respectively, after the commencement of the injections. The urines contained much albumin, but no crystals. At autopsy the livers showed marked passive congestion and degenerative fatty infiltration. The renal tubules of three animals contained eosinophilic material infiltrated with polymorphonuclear leucocytes. The bone marrow was cellular and did not differ from that of the animals previously described. Reticulocytosis did not occur.

Little stress can be laid on the blood counts in these guinea-pigs, owing to their very bad general condition, and the fact that they became unable to drink water or to eat.

Four animals were given 50 mg. pteroylglutamic acid alone by injection. Some loss of weight occurred, but the animals did not become severely ill like those just described.

Effects/

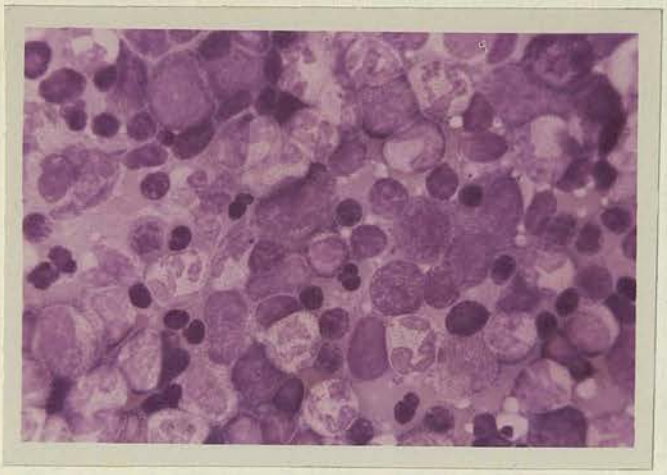
Effects of Succinylsulphathiazole in Addition to 4-aminopteroylglutamic Acid. Production of a more severe macrocytic Anaemia.

Further experiments were carried out to assess the effect of adding succinylsulphathiazole in powder form to the diet in the proportion of 2%. The intestion was in this way to prevent possible intestinal synthesis of folic acid by bacteria.

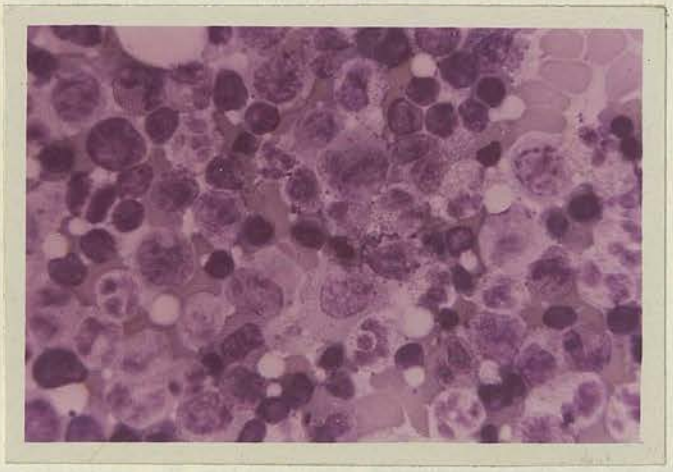
Succinylsulphathiazole alone was administered to 8 animals for a period of 5 weeks and did not produce anaemia or other adverse effect.

Five animals were made anaemic by the giving of 0.25 mg. 4-aminopteroylglutamic acid daily for 5 weeks and, when there appeared to be some recovery from this anaemia, succinylsulphathiazole (2%) was added to the diet. (The reason for this recovery is unexplained, but the phenomenon has been noted by several workers using guinea-pigs.) As will be seen from Table 18, the anaemia became more severe when the succinylsulphathiazole was added.

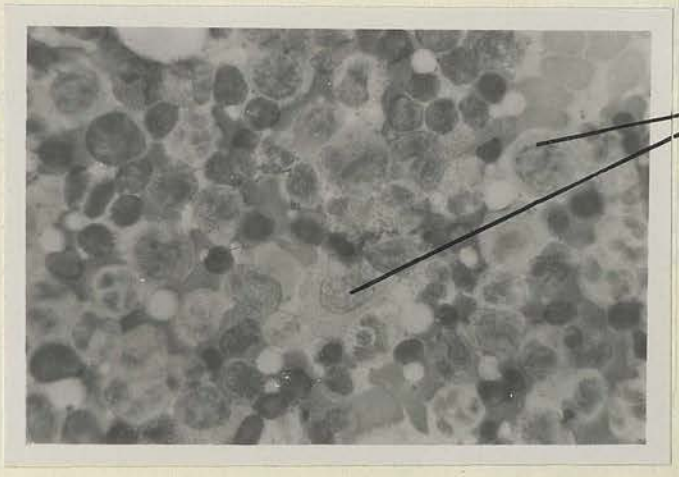
Within a few days of the addition of the succinylsulphathiazole the animals, which had previously appeared to be healthy, became lethargic, refused to eat, and began to lose weight rapidly. The hair became rough and marked generalised oedema developed. There was individual variation in the response, however. One died in 7 days and another in 14 days.



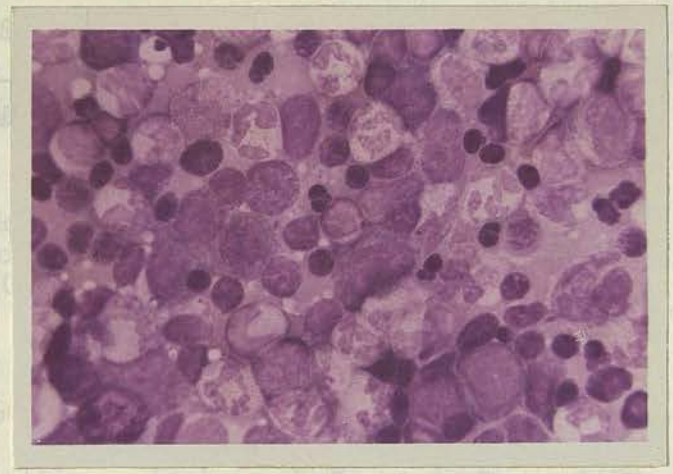
Bone Marrow of Normal Guinea Pig (Wright's stain.)



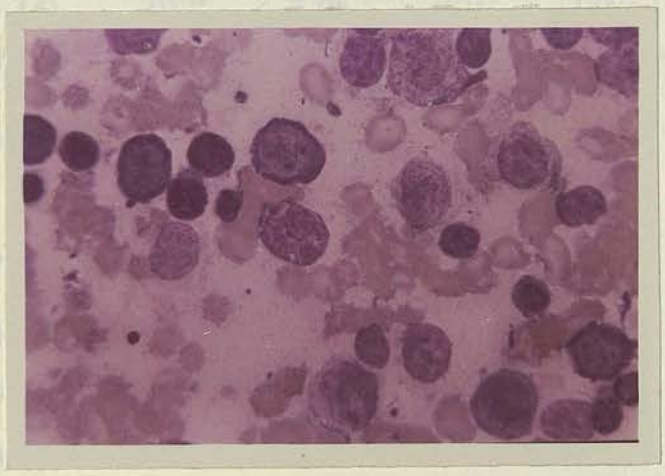
Bone Marrow of guinea pig treated with 0.25 mg. of 4-aminopteroylglutamic acid daily for seven weeks.



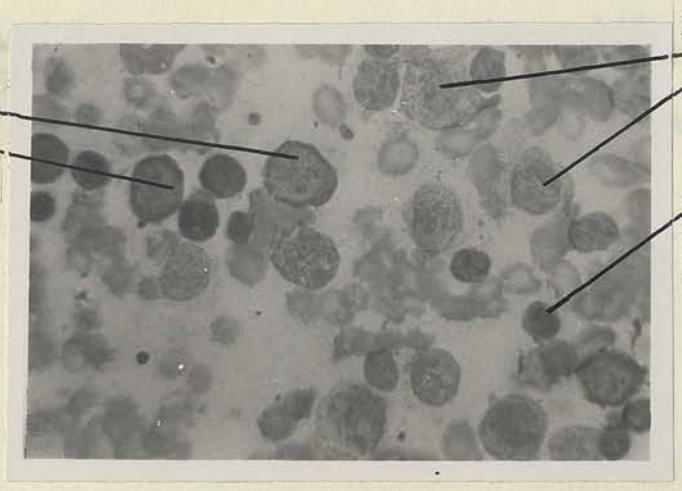
Key to the second picture shown above.



Bone Marrow of Normal Guinea Pig. (Wright's stain.)



Bone Marrow of guinea pig treated with 0.25 mg. of 4-aminopteroylglutamic acid daily for seven weeks but with succinylsulphathiazole in the diet.



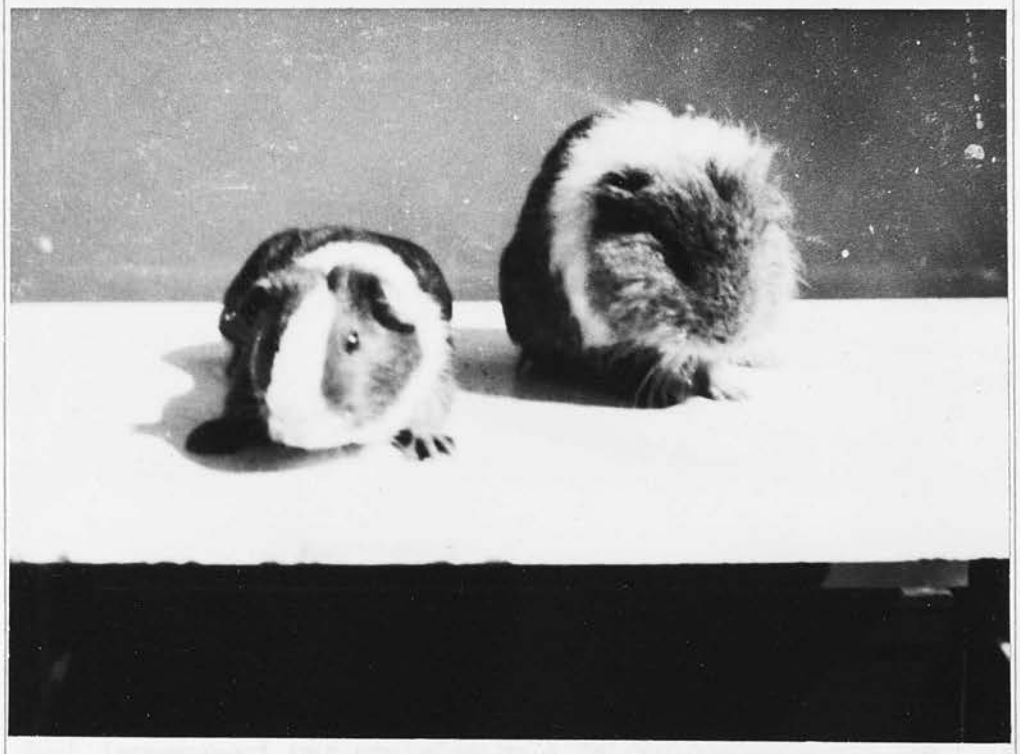
Key to the second picture shown above.
(This field was less cellular than the average one.)

TABLE 18.

Effects on Blood Picture of adding 2% Succinylsulphathiazole to the Diet of Guinea pigs already receiving 0.25 mg. 4-aminopteroylglutamic acid daily.

Time (wks.)	Mean Weight (G.)*	Hb. C./ 100 ml.	R.B.C. mills/ c.mm.	W.B.C. per c.mm.	Polys. per c.mm.	P.C.V. (%)	M.C.V. (cμ.)
			4-Aminopteroylglutamic acid given alone				
0	576 (5)	13.0	5.04	6030	1780	41.8	83.1
1	851 (5)	12.1	4.30	5730	1780	37.9	88.5
2	590 (5)	11.1	4.06	5670	1910	36.8	91.6
3	555 (5)	10.7	3.96	4010	1240	35.1	89.3
4	540 (5)	8.7	3.32	2450	560	30.2	91.6
5	543 (5)	9.8	3.51	4310	1350	31.3	90.4
			Succinylsulphathiazole added to diet				
6	497 (5)	8.4	3.17	2810	1150	28.8	91.8
7	484 (4)	7.5	2.46	5180	2260	26.0	106.8
8	590 (3)	7.1	2.45	8530	4280	26.8	108.6
9	599 (3)	7.5	2.85	7010	2730	28.0	100.8
10	627 (3)	8.1	3.23	7350	2930	30.8	97.1
11	629 (3)	8.6	3.38	6280	3600	31.2	96.2
12	645 (3)	6.9	2.40	3820	1330	25.6	109.6

* Figures in parentheses indicate the number of animals alive at this time.



The guinea-pig on the left has been given 0.25 mg. 4-aminopteroylglutamic acid for 7 weeks. That on the right has had, in addition, 2% succinylsulphathiazole in its diet for two weeks.

Severe anaemia developed in all five animals, the red cell count falling below 2,000,000/cu.mm. at some stage in the three animals that survived the experiment. Two animals then showed definite haematological improvement, but this was not maintained. So far as general effects were concerned, the three animals that survived the first results of the addition of succinylsulphathiazole to their diet, while the administration of 4-aminopteroylglutamic acid was continued, then showed marked improvement, becoming active again, eating well, gaining weight, and looking like normal animals. One such animal had a fall in the red cell count to 790,000/cu.mm., but only the pallor of the mucous membranes indicated that the animal/

animal was abnormal. The serum was not bile stained even when anaemia was severe.

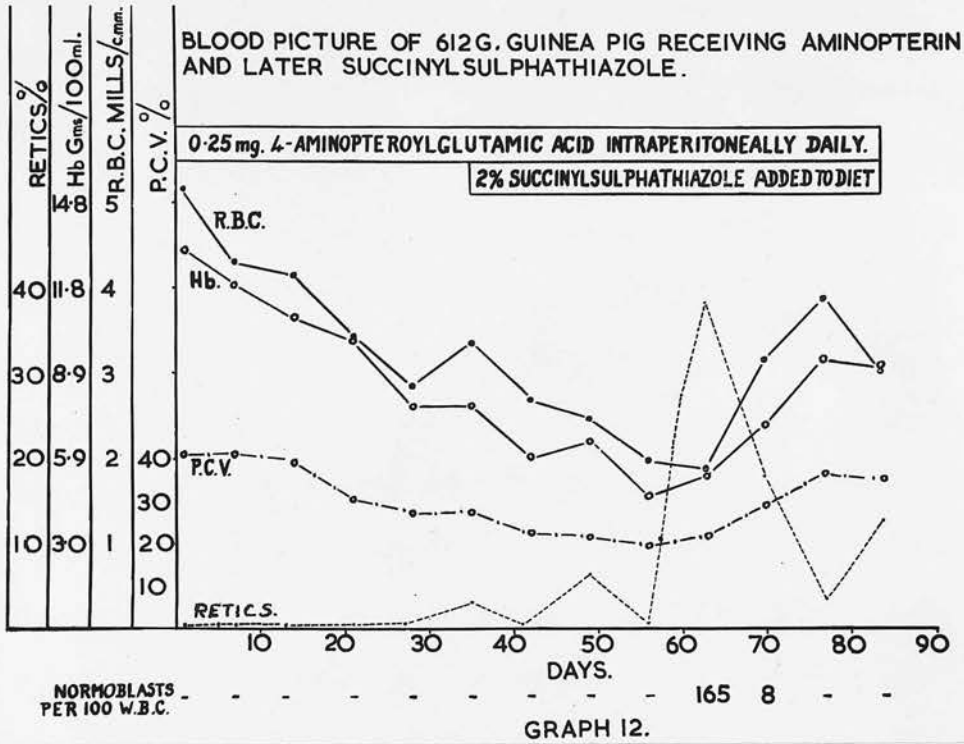
This degree and type of anaemia could not be produced by an increase of the dose of 4-aminopteroylglutamic acid alone, since the administration of even 4 mg. daily would not produce it (Innes et al., 1949). Minnich & Moore (1948) found that continued administration of 0.5 mg. - 5.0 mg. daily caused death. At autopsy in their animals the marrow was aplastic.

As has been mentioned above, the animals receiving small doses of 4-aminopteroylglutamic acid together with succinylsulphathiazole tended to show temporary haematological improvement even in the absence of any change in their treatment. Such improvement was preceded by a reticulocytosis and the appearance of normoblasts in the peripheral blood. The serum was not bile stained. When the anaemia was most severe the polymorphonuclear leucocytes showed toxic granulation.

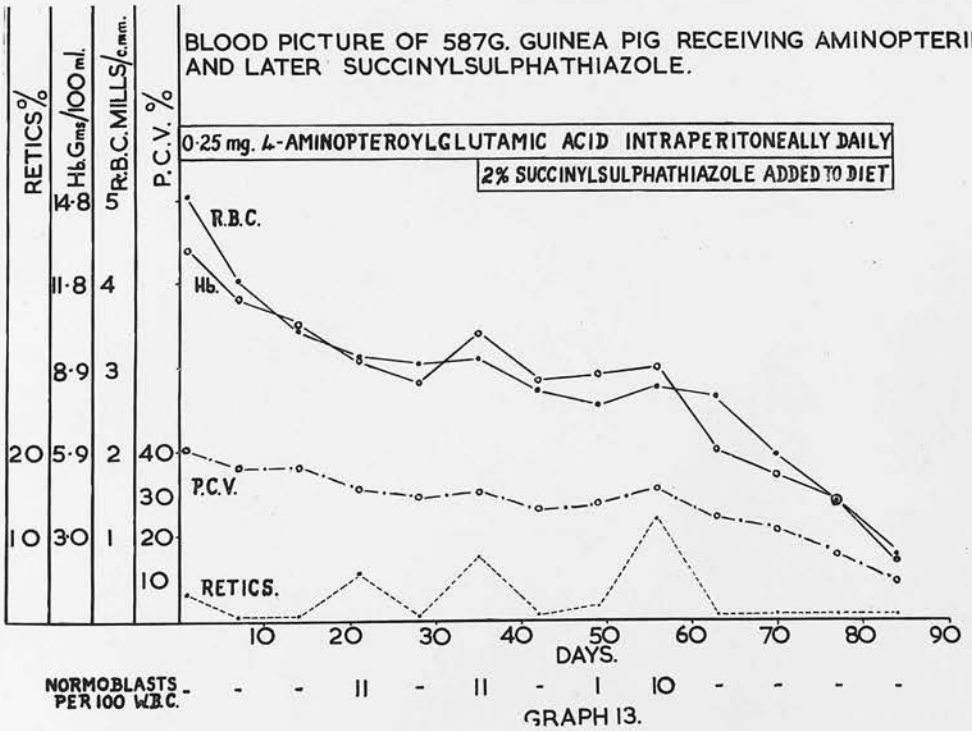
It will be seen that the mean corpuscular volume reached a higher value in this group than in any other animals, and this was not explained merely by the presence of large numbers of reticulocytes.

In Graphs 12 and 13 there are given the blood findings in two guinea pigs treated with 4-aminopteroylglutamic acid and succinylsulphathiazole. The varying responses are shown.

Examination after death or after the animal had been killed showed that the marrows were cellular and contained an increase in megakaryocytes. Haemocytoblasts and proerythroblasts were very numerous and this was the outstanding feature of the marrow picture. In this respect the marrow simulated that/



GRAPH 12.



GRAPH 13.

that of severe untreated pernicious anaemia in man. There were, too, some cells with a certain degree of similarity to the intermediate and late megaloblasts of man, and again, a marked increase in reticulum cells. The cells that simulated the intermediate megaloblast had a more open-work nucleus than the intermediate normoblast of the normal animal. More numerous than the former were poorly haemoglobinised cells with megaloblast-like nuclei and a bluish rim of cytoplasm. Intermediate and late normoblasts were very few. A typical differential marrow count is given in Table 19.

In summary the striking feature of the marrow was a great increase in reticulum cells, proerythroblasts and haemocytoblasts, together with a diminution in neutrophil polymorphs, and in intermediate and late normoblasts, and the development of some cells with a certain resemblance to the megaloblast.

So far as the viscera were concerned, fatty infiltration of the liver occurred in some instances. The small intestine did <u>not</u> show atrophy in any.

Representative autopsy findings on four animals given 4-aminopteroylglutamic acid for six weeks with the <u>addition of</u> succinylsulphathiazole thereafter are as follows:

<u>Guinea pig N2</u>. Died two weeks after succinylsulphathiazole commenced. Hb. then 8.4 G./100 ml., red cells 2,960,000 per c.mm., PCV 32%, MCV 108.6 cμ., Reticulocytes 9%, WBC 7,000, Polymorphs 5,320 per c.mm. (toxic).
<u>Liver</u>: Very marked fatty infiltration and degenerative changes.
<u>Lung</u>: Acute purulent lobular pneumonia. Abundant lymphoid tissue in peribronchiolar walls. Hypertrophic serosal cells in visceral pleura.
<u>Spleen</u>: Focal chronic productive perisplenitis. Mild haemosiderosis of the pulp.
<u>Adrenal</u>: Abundant cortical lipids.
<u>Kidneys</u>:/

Kidneys: Many of the collecting tubules show vacuolar change and simple necrosis. Moderate degenerative fatty infiltration of renal tubular epithelium in fat stained sections.

Large Intestine: Slight to moderate irregular atrophy of the mucosa and the musculature. Chronic peritonitis.

Small Intestine: Focal chronic peritonitis. Haemosiderin containing phagocytes in the submucosa.

Summary: The main features were lipoid changes in the liver and kidney. Some peritonitis.

Marrow: Cellularity about 95%. Numberous haemocytoblasts and early precursor cells of the red cell series. Increase in myeloblasts and in reticulum cells. Few megaloblast-like cells.

Guinea pig N3. Died one week after succinylsulphathiazole commenced. Hb. then 9.9 G./100 ml., red cells 3,845,000 per c.mm., PCV 37.5%, MCV 98.0 cμ., Reticulocytes < 1%, WBC 3,200, Polymorphs 1,600 per c.mm.

Liver: Multiple foci of simple necrosis, with mild infiltration of polymorphonuclear leucocytes. The necrosis appears to be predominantly midzonal in distribution. Fat stain shows no abnormality.

Lungs: Marked acute passive congestion. Increase of lymphocytes around blood vessels and in alveolar walls. Patchy alveolar haemorrhages by diapedesis.

Spleen: Atrophy. The vascular elements were quite reduced in number in the sinusoids. Haemosiderin-bearing phagocytes were prominent in the pulp.

Adrenals: Abundant cortical lipids in fat stain.

Kidneys: Moderate hydropic degeneration of tubular epithelium. Passive congestion.

Large Intestine: Slight mucosal atrophy.

Small Intestine: Brown pigmentation of the mucosa.

Summary: The main feature is hepatic necrosis.

Marrow: Cellularity about 70%. Numerous haemocytoblasts and early red cell precursors. Increase in myeloblasts and in reticulum cells. Few megaloblast-like cells. Adult polymorphs reduced.

Guinea/

Guinea pig N4. Killed seven weeks after succinylsulphathiazole commenced. Hb. then 9.0 G./100 ml., red cells 3,000,000 per c.mm., PCV 34.5%, MCV 115.0 cμ., Reticulocytes 12.5%, WBC 4,750, Polymorphs 880 per c.mm. (See also Graph 12). Animal active.

Liver: Mild degenerative fatty infiltration of hepatic cells.
Lungs: Emphysema. Moderate sclerosis of medium sized pulmonary vessels.
Spleen: Passive congestion. Moderate number of haemosiderin-containing phagocytes in the pulp.
Adrenals: Mild reduction of cortical lipids in sections stained with fat stain.
Kidneys: Passive congestion. Mild degenerative fatty infiltration of renal tubular epithelium.
Large Intestine: Abundant mucin. Brown pigmentation of mucosa.
Small Intestine: No abnormality.

Summary: Some fatty infiltration of liver and kidneys.

Marrow: Cellularity about 70%; great increase in haemocytoblasts and proerythroblasts. Abundant megakaryocytes. Increase in myeloblasts and in reticulum cells. A number of megaloblast-like cells but these were only about 2% of the red cell precursors. Few adult polymorphs.

Guinea pig N5. Killed seven weeks after succinylsulphathiazole commenced. Hb. then 2.1 G./100 ml., red cells 790,000 per c.mm., PCV 9.0%, MCV 113.9 cμ., Reticulocytes 1%, WBC 2,050, Polymorphs 480 per c.mm. (See also Graph 13) Animal active.

Liver: Very marked fatty infiltration and degenerative changes.
Lungs: Abundant peribronchiolar lymphoid tissue. Moderate emphysema.
Spleen: Distension and passive congestion of the sinusoids. Slight fibrous thickening of the capsule. Occasional megakaryocyte seen in sinusoids.
Adrenals: Brown pigmentation of inner cortex. Abundant cortical lipids demonstrated with fat stain.
Kidneys: Moderate degenerative fatty infiltration of tubular epithelium.
Large Intestine: Brown pigmentation of the mucosa.
Small Intestine: No abnormality.

Summary:/

TABLE 19.

Bone Marrow Cells in Guinea Pigs.

	Normal Guinea Pig	Treated with 0.25mg. 4-amino-PGA	Treated with 0.25 mg. 4-amino-PGA and sulphasuccidine (Animal N5)
Reticulum cells	-	1.0	36.0
* Proerythroblasts	-	4.2	15.0
Early normoblasts	4.0	2.5	13.8
Early megaloblasts	-	-	-
Intermed. normoblasts	23.0	20.2	7.5
Intermed.megaloblasts	-	-	0.2
? Hypochromic intermed. megaloblasts	-	-	6.0
Late normoblasts	21.0	18.1	6.0
Late megaloblasts	-	-	0.3
Neutrophil (Heterophil)			
Myelocytes	4.0	8.5	4.0
Metamyelocytes	-	-	-
Polymorphs	34.4	37.5	0.5
Eosinophil			
Myelocytes	2.0	1.0	1.5
Polymorphs	7.0	7.0	-
Basophil			
Myelocytes	0.2	-	-
Polymorphs	1.0	-	-
Premyelocytes	-	-	1.0
Myeloblasts	-	-	1.0
Lymphocytes	-	-	7.1
Plasma cells	-	-	-
Megakaryocytes	-	-	0.1
Monocytes	-	-	-
Unidentified	3.4	-	-

* (Haemocytoblasts are included under this heading)

Summary: Lipoid changes in liver and kidneys.

Marrow: Cellularity about 50%: great increase in haemocytoblasts and proerythroblasts. Abundant megakaryocytes. Increase in myeloblasts and in reticulum cells. About 12% of the red cell precursors were megaloblast-like cells. Very few adult polymorphs.

Protection by Pteroylglutamic Acid.

This last experiment was repeated, but 2.5 mg. pteroylglutamic acid were given daily intraperitoneally in addition to 0.25 mg. 4-aminopteroylglutamic acid.

It will be seen from Table 20 that worsening of the anaemia did not occur when succinylsulphathiazole was added to the diet of this group of guinea pigs. The general appearance of the animals at the end of the experiment was similar to that of guinea pigs that received only 0.25 mg. 4-aminopteroylglutamic acid daily without succinylsulphathiazole.

In a further experiment, summarised in Table 21, two groups of five animals were given 0.25 mg. 4-aminopteroylglutamic acid and 2% succinylsulphathiazole in the diet from the commencement. One of the groups received, in addition, 5 mg. pteroylglutamic acid daily. Again the effect of succinylsulphathiazole in increasing the anaemia was overcome by pteroylglutamic acid. At the end of 7 weeks, three animals with low reticulocyte counts had red cell values of less than 1,500,000/cu.mm., whereas another guinea pig of similar weight had a count of 4,205,000/cu.mm. associated with a reticulocytosis of 23.5%. The general features and bone marrow changes were as before. The two most anaemic animals received daily, by injection, 5 mg. pteroylglutamic acid at the end of the seventh week. This was followed by/

TABLE 20.

Effects on blood picture of adding 2% succinylsulphathiazole to the Diet of five Guinea pigs already receiving 0.25 mg. 4-aminopteroylglutamic acid and 2.5 mg. pteroylglutamic acid daily. (The administration of the last two was continued throughout).

Time (Wks.)	Mean Weight (G.)	Hb. G./ 100 ml.	R.B.C. mills/ c.mm.	W.B.C. per c.mm.	Polys. per c.mm.	P.C.V. (%)	M.C.V. (cμ.)
4-Aminopteroylglutamic acid and Pteroylglutamic Acid given							
0	426	13.4	5.28	8360	4620	46.0	87.6
1	443	11.3	4.43	6960	3880	39.3	91.0
2	476	11.6	4.24	5370	2760	39.1	95.4
3	494	10.7	4.27	5440	2510	37.9	89.6
4	536	10.3	3.87	4190	1400	33.6	86.8
5	566	10.1	4.26	7160	3700	35.4	83.6
Succinylsulphathiazole added to diet							
6	544	9.7	3.95	4840	940	33.7	85.6
7	589	10.1	3.99	3670	1240	31.9	80.6
8	608	10.0	4.08	5910	2220	32.5	80.0

TABLE 21.

Effects on blood picture of Administration to Groups of five Guinea pigs of 0.25 mg. 4-aminopteroylglutamic acid daily together with Succinylsulphathiazole with and without Pteroylglutamic Acid

Time (Wks.)	Mean Weight (G)	Hb. G./ 100 ml.	R.B.C. mills/ c.mm.	W.B.C. per c.mm.	Polys. per c.mm.	P.C.V. (%)	M.C.V. (cμ.)	
0	408	13.5	5.32	8920	5030	46.3	87.3	
1	432	12.9	4.61	7500	4230	38.8	84.8	
2	446	9.1	4.26	5860	2620	39.1	92.2	4-aminopteroyl-glutamic acid given
3	474	10.9	4.21	5550	3100	36.8	91.1	
4	487	10.4	4.24	7230	2100	37.1	88.5	
5	518	11.2	4.51	8430	4500	39.3	80.7	
6	522	10.2	3.72	4710	2530	33.2	88.7	
7	529	9.9	4.14	4880	2870	35.3	86.1	
0	385	12.2	4.95	6970	3550	44.3	89.7	
1	408	12.0	4.52	5640	2870	40.9	91.1	
2	393	11.3	4.38	5840	1750	38.0	87.0	4-aminopteroyl-glutamic acid & succinylsulpha-thiazole given
3	410	8.9	3.15	5860	2060	30.8	100.6	
4	435	8.3	3.01	4420	1440	26.6	89.8	
5	451	7.0	2.49	3960	600	23.5	95.7	
6	473	6.8	2.34	3010	890	23.3	100.2	
7	462	6.4	2.10	2450	490	21.3	101.6	
0	385	12.7	5.11	5900	2340	42.9	84.1	
1	389	11.2	4.28	4330	2060	37.9	89.9	
2	395	10.6	3.92	5670	1830	35.0	89.7	4-aminopteroyl-glutamic acid, pteroylglutamic acid & succinyl-sulphathiazole given
3	399	10.1	3.67	4350	1170	33.9	93.7	
4	408	10.1	3.44	6870	3100	32.4	95.1	
5	437	9.5	3.43	4480	1150	32.1	95.1	
6	469	9.7	3.51	5160	1760	33.6	96.9	
7	486	9.1	3.78	5260	1650	31.9	82.5	

by reticulocytosis and a rise in the red cell count.

DISCUSSION OF THESE RESULTS.

In guinea-pigs of suitable weight, 0.25 mg. 4-amino-pteroylglutamic acid daily caused moderate anaemia of macrocytic type with a cellular marrow that showed an increase in early red cell precursors and in reticulum cells. The effects were not prevented by pteroylglutamic acid, thymine, para-amino-benzoic acid, or by liver preparations employed in the manner described. Citrovorum factor preparations were not available.

When the daily dose of 4-aminopteroylglutamic acid was increased, the anaemia became more severe, but general toxic effects also increased. It has been shown by other workers (Innes et al., 1949) that a single dose of even 40 mg. has little effect. It is the daily administration of the drug that leads to anaemia, but if 2-5 mg. is given daily, aplastic anaemia develops. (Minnich & Moore, 1948; Innes et al., 1949; Messerschmitt, 1949). Increasing the dosage of pteroylglutamic acid did not prevent the anaemia producing effects of 4-amino-pteroylglutamic acid and, indeed, the combination of 0.5 mg. of this substance with 50 mg. of pteroylglutamic acid was lethal to the guinea-pig.

The giving of succinylsulphathiazole in addition to 4-aminopteroylglutamic acid caused a more severe macrocytic anaemia with leucopenia and the appearance in the marrow of large numbers of haemocytoblasts and proerythroblasts together with/

with a larger proportion of reticulum cells and also a few cells with a certain degree of resemblance to the intermediate and late megaloblasts in man. A noticeable feature was the marked fall in the number of cells of the granulocyte series despite the number of red cell precursors present. It was possible to produce animals with very severe anaemia but no other obvious clinical abnormality if they survived the commencement of the administration of this combination of drugs.

The intention was to produce folic acid deficiency by two different methods, firstly by using 4-aminopteroylglutamic acid to interfere with folic acid metabolism, and secondly by using succinylsulphathiazole in an attempt to prevent possible synthesis of folic acid by intestinal bacteria. The results were interesting in that pteroylglutamic acid, given by injection, prevented the aggravation of the anaemia by succinylsulphathiazole but did not prevent the anaemia producing effect of 4-aminopteroylglutamic acid. The livers of the various animals were assayed microbiologically for their content of vitamin B_{12} (Table 22). No reduction occurred when 4-aminopteroylglutamic acid was given alone or with succinylsulphathiazole so that it appears reasonable to assume that the anaemia was not produced by deficiency of vitamin B_{12}. Assays of the livers for folic acid were invalidated by the fact that a folic acid antagonist had been given.

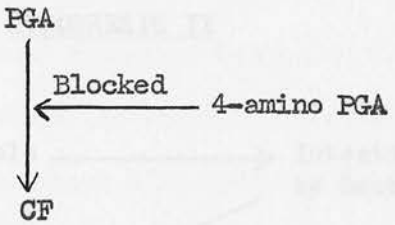
Since the aggravation of the anaemia by succinylsulphathiazole could consistently be prevented or reversed by pteroylglutamic acid, the most likely possibility is that the sulphonamide/

TABLE 22.

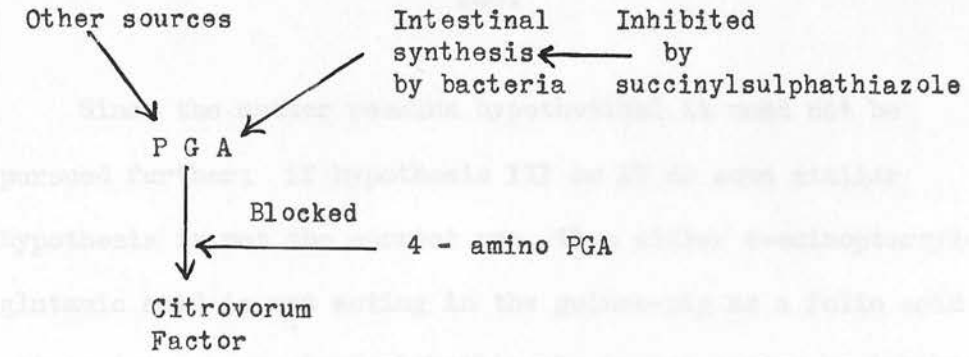
Liver Content of Growth Factors for Lb.leichmannii in various Groups of Guinea-pigs

Group	No. of animals	Lb.leichmannii growth factors as vitamin B_{12} (µg./G.) Mean	Range
Normal controls	5	0.16	(0.08-0.31)
Receiving 0.25 mg. 4-amino-pteroylglutamic acid daily	10	0.34	(0.14-0.76)
Receiving 0.25 mg. 4-amino-pteroylglutamic acid and 0.45 G. thymine daily	5	0.34	(0.24-0.43)
Receiving 0.25 mg. 4-amino-pteroylglutamic acid and 2.5 mg. pteroylglutamic acid daily	5	0.49	(0.25-0.77)
Receiving 0.25 mg. 4-amino-pteroylglutamic acid and 2% succinylsulphathiazole daily	5	0.33	(0.19-0.48)

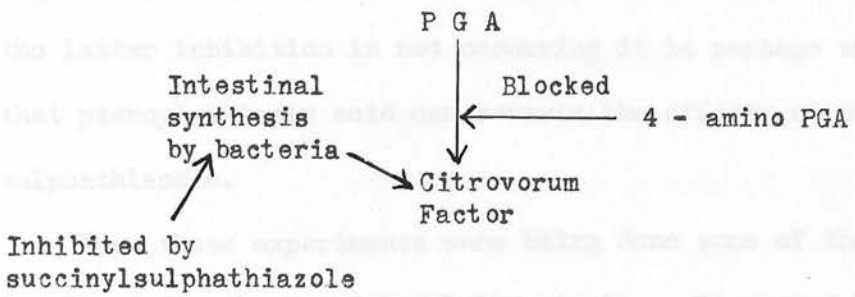
sulphonamide is acting by inhibiting intestinal bacteria that can synthesise folic acid or citrovorum factor. Evidence that certain micro-organisms can carry on such a synthesis has been given by numerous workers including Hutchings et al. (1941), Mitchell & Isbell (1942) and Thompson (1942). On the other hand the action of 4-aminopteroylglutamic acid could <u>not</u> be prevented by pteroylglutamic acid. As has already been said, the general consensus of opinion now is that the <u>main</u> action of 4-aminopteroylglutamic acid is to prevent the conversion of pteroylglutamic acid to citrovorum factor thus:



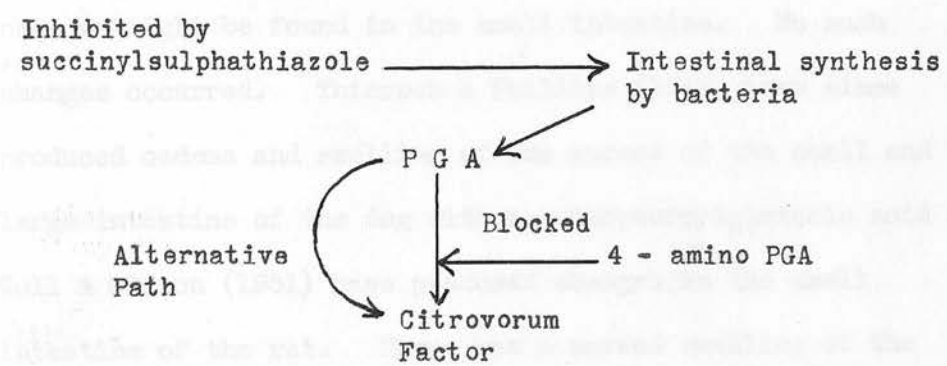
If this is so, and as the action 4-aminopteroylglutamic acid cannot be reversed by pteroylglutamic acid, then the present results may indicate the existence of another pathway for the conversion of pteroylglutamic acid to citrovorum factor. Hypotheses I and II would not explain the experimental results, but hypothesis III or IV would do so. (p.165a)



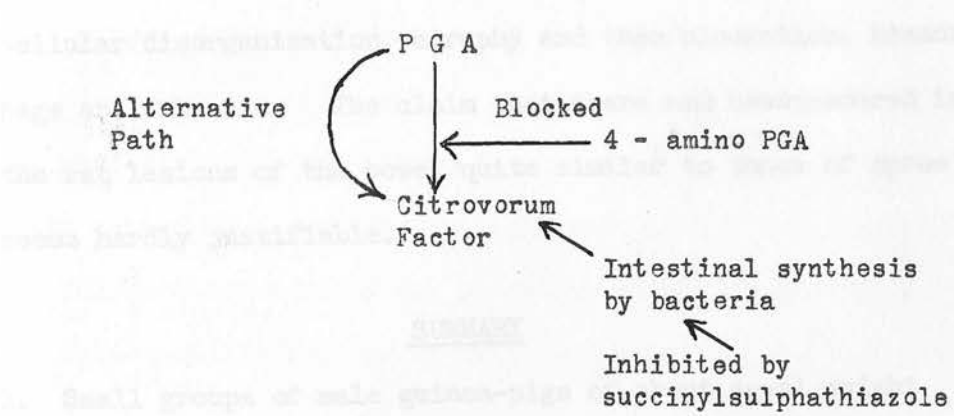
HYPOTHESIS I



HYPOTHESIS II



HYPOTHESIS III



HYPOTHESIS IV

Since the matter remains hypothetical it need not be pursued further; if hypothesis III or IV or some similar hypothesis is not the correct one, then either 4-aminopteroylglutamic acid is not acting in the guinea-pig as a folic acid antagonist, or succinylsulphathiazole is not acting by inhibiting the synthesis of folic acid by intestinal organisms. If the latter inhibition is not occurring it is perhaps surprising that pteroylglutamic acid can reverse the effects of succinylsulphathiazole.

When these experiments were being done some of the animals developed diarrhoea and had bulky stools. It seemed possible that a form of sprue was being produced and that histological changes might be found in the small intestine. No such changes occurred. Thiersch & Phillips (1949) have since produced oedema and swelling of the mucosa of the small and large intestine of the dog with 4-aminopteroylglutamic acid and Woll & Oleson (1951) have produced changes in the small intestine of the rat. There was a marked swelling of the cytoplasm and nucleus of the cells of the glands, followed by cellular disorganisation, atrophy and then ulceration, haemorrhage and fibrosis. The claim that there had been produced in the rat lesions of the bowel 'quite similar to those of sprue' seems hardly justifiable.

SUMMARY

1. Small groups of male guinea-pigs of about equal weight developed a mild macrocytic anaemia when they were given 0.25 mg. 4-aminopteroylglutamic acid daily. This action was not prevented by simultaneous administration of pteroylglutamic/

pteroylglutamic acid in quantities ten or twenty times as great, or by giving thymine, p-aminobenzoic acid or liver by mouth or injection. The marrow contained an increased number of early red and white cell precursors and also numerous reticulum cells.

2. The addition of succinylsulphathiazole to the diet in the proportion of 2% resulted in a more severe form of macrocytic anaemia when 4-aminopteroylglutamic acid was given simultaneously in the above dosage. This aggravation of the anaemia was prevented by giving 2.5 mg. pteroylglutamic acid daily. The marrow of these more anaemic animals contained very many reticulum cells, haemocytoblasts and proerythroblasts, and a few cells that had some resemblance to the megaloblast series in man, but were not identical with them in appearance. On the other hand, they differed morphologically from cells of the normoblast series in the guinea pig. Cells of the granulocyte series became much diminished in numbers.

3. There were fatty changes in the liver. No constant lesions occurred in the small intestine to suggest that there had been produced an experimental sprue syndrome with histological changes in the gut.

4. It seemed that 4-aminopteroylglutamic acid and succinylsulphathiazole might not be acting merely by producing folic acid deficiency in different ways.

CHAPTER 4.

MICROBIOLOGICAL ASSAY METHODS EMPLOYED IN THE INVESTIGATIONS THAT FOLLOW.

The measurement of small quantities of cyanocobalamin, pteroylglutamic acid or citrovorum factor is carried out by microbiological assay methods. Taking cyanocobalamin as an example, the procedure when the tube assay is done is as follows:

A number of test tubes are partly filled with a measured quantity of a suitable medium, and graded dilutions of crystalline cyanocobalamin in solution.

In further series of tubes there is put the same amount of the same medium, and graded dilutions of the substance to be tested (or a solution or liquid extract of the substance). The tubes are then autoclaved.

The contents of all the tubes (other than one uninoculated blank) are inoculated with the test organism, in this instance L.leichmannii or a mutant of B.coli, which grows only in the presence of cyanocobalamin.

The tubes are incubated, e.g. for 16 hours.

The amount of growth will depend upon the amount of cyanocobalamin present, and so the turbidity of the tubes containing the unknown is compared with a standard curve made up from photoelectric readings of the turbidity of the organisms growing in/

in the tubes containing crystalline cyanocobalamin.

From these readings the amount of vitamin B_{12} in the unknown can be calculated.

All readings are done in triplicate.

The techniques, unfortunately, are complicated and the difficulties are many. Nevertheless, as will be seen from the recovery experiments recorded in later chapters, the results are surprisingly accurate. In the present chapter there is given only a summary of the techniques that we evolved in the course of several years of experimentation. Since 1952 we have used several alternative methods for the cyanocobalamin assays, as follows:

Lactobacillus leichmannii Assay for Cyanocobalamin.

Composition of Medium No.1 for L.leichmannii Assay

Constituent	Amount for 100 ml. of double strength medium
Glucose, anhydrous	2.0 G.
Sodium acetate, anhydrous	2.0 G.
*Peptone solution	5.0 ml.
Salt A	2.0 ml.
Salt B	2.0 ml.
Tween 80	0.2 ml.
l-asparagine	20.0 mg.
Dl-tryptophane	40.0 mg.
l-cystine	40.0 mg.
Adenine sulphate	2.0 mg.
Guanine sulphate	2.0 mg.
Uracil	2.0 mg.
Xanthine	2.0 mg.
p-aminobenzoic acid	250.0 µg.
Biotin	0.4 µg.
Pteroylglutamic acid	4.0 µg.
Nicotinic acid	200.0 µg.
d-calcium pantothenate	50.0 µg.
Pyridoxine hydrochloride	50.0 µg.
**Pyridoxal	50.0 µg.
Aneurin hydrochloride	50.0 µg.
Riboflavin	100.0 µg.

* Supplied by the kindness of Mr. G.E.Shaw of Evans Biological Institute.

** Obtained from the United States.

The medium is modified from that described by Hoffmann et al. (1949).

1. The pH of this medium was adjusted to 6.8.

2. The medium was not kept for longer than one week, in a refrigerator at 4°C.

3. Reducing agents were not included. The medium was autoclaved separately and the cyanocobalamin of the standard added aseptically. The substances being tested were also sterilised and added aseptically to the medium.

4. The medium was autoclaved in the tubes in a pressure cooker for 10 minutes at 15 lbs. pressure. Each tube contained 5 ml. of medium. The pressure was lowered quickly and the tubes cooled in cold water.

5. The final total volume in each tube was 10 ml. There were seven tubes in the rows of standard cyanocobalamin, containing respectively 0, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mμg. of cyanocobalamin. The solution to be tested was adjusted to contain an amount of vitamin B_{12} of this order of magnitude, and was added to five tubes in volumes of 1, 2, 3, 4, and 5 ml. together with 4, 3, 2, 1 and 0 ml. of water respectively.

6. After inoculation with L.leichmannii the tubes were incubated at 37°C. for 16-18 hours, and growth then read by measurement of turbidity in a photoelectric absorptiometer.

7. Since thymidine and other desoxyribosides are stable to hydrolysis with dilute alkali, whereas cobalamin compounds are inactivated under these conditions, a correction was made as follows:-

   To the samples to be tested there was added Na OH so that the alkali was present in the final volume as 0.2 N Na OH. This mixture was then heated for 30 minutes in a steam bath. Such treatment destroys only vitamin B_{12} and other cobalamin compounds, whereas at a higher pH some desoxyribosides may be destroyed.

   After such treatment the pH was adjusted to 6.8 and an assay carried out in the usual way with L.leichmannii. This gave the amount of vitamin B_{12} activity that was really due to the presence of desoxyribosides.

8. Salt mixture A contains

$K_2 HPO_4$		100 mg.
$KH_2 PO_4$		100 mg.
Water	to	1 ml.

Salt/

Salt mixture B contains	$MgSO_4\ 4H_2O$		40 mg.
	NaCl		2 mg.
	$FeSO_4\ 4H_2O$		2 mg.
	$MnSO_4\ 4H_2O$		2 mg.
	Water	to	1 ml.

9. L.leichmannii 313 was used as the test organism.

Composition of medium No.2 for L.leichmannii assay.

This was similar to medium number 1, except that the peptone solution was omitted and replaced by Allen & Hanbury's acid hydrolysed casein, 1 G. being included per 100 ml. of double strength medium. Thioglycolic acid was added in a proportion of 40 mg. per 100 ml. of double strength medium. The assay was carried out as before except for the important difference that the medium and standard cyanocobalamin or solution to be assayed were autoclaved together - i.e. an aseptic technique was not employed. It was for this reason that thioglycolic acid was included, since Hoffmann et al. (1949) claimed that reducing agents of this nature 'protected' cyanocobalamin from destruction when it is autoclaved with the medium.

For experiments with serum, the serum was added aseptically to avoid precipitation of proteins by heat.

Composition of medium No.3 for L.leichmannii assay.

In a later chapter there are described experiments designed to estimate the amount of vitamin B_{12} present in the serum. In the earlier experiments medium No.2 was used but as will be described in the relevant chapter, a third medium was eventually evolved because the preliminary treatment of the serum involved/

involved the use of acetate and the amount of acetate in the medium had to be reduced to avoid inhibition of growth of the test organism.

	Amount for 100 ml. of double strength medium
Casein (hydrolysed)	1 G.
Dl-tryptophane	20 mg.
l-Cystine	20 mg.
Adenine sulphate	1 mg.
Guanine sulphate	1 mg.
Uracil	1 mg.
Xanthine	1 mg.
Salts A	1 ml.
Salts B	1 ml.
Sodium Acetate (anhydrous)	1.2 G.
Glucose	4 G.
Biotin	1 µg.
Pyridoxine hydrochloride	400 µg.
Pyridoxal	400 µg.
Riboflavin	200 µg.
Aneurin hydrochloride	200 µg.
Ca. pantothenate	200 µg.
Nicotinic acid	200 µg.
Pteroylglutamic acid	100 µg.
Thioglycolic acid	40 mg.
Tween 80	0.2 ml.

The pH was adjusted to 6.8.

In the earlier experiments thioglycolic acid was not included, but ascorbic acid 200 mg. and fumaric acid 100 mg. were added. This gave unduly high blanks. The medium is modified from that of Thompson et al. (1950).

Bacillus coli assay for cyanocobalamin.

The L.leichmannii assay is not specific for cyanocobalamin. In the absence of this substance growth may occur with thymidine, adenine desoxyriboside, cytosine desoxyriboside, or hypoxanthine desoxyriboside, but the method of correcting for this by/

by alkaline hydrolysis has already been described. In addition certain other growth factors for L.leichmannii have been isolated from the gut contents or from faeces (Holdsworth & Ford, 1953; Ford et al., 1953). There are four main factors of this type: they are not cobalamins and cannot be converted into such. To them have been given the names factor A (vitamin B_{12m}), factor B, factor C and pseudovitamin B_{12}. So far these substances have only been shown to be of importance in attempts to measure the content of vitamin B_{12} in the faeces or in the intestinal contents and each one of them differs in its activity for the various organisms used in testing for vitamin B_{12} activity. (See p.202).

In the present series of investigations the only method used for cyanocobalamin assays other than the L.leichmannii technique was one employing an ultra violet ray induced mutant of B.coli which requires cyanocobalamin for growth. This method was first described by Davis & Mingioli (1950). Unlike L.leichmannii, this organism will not use desoxyribosides as growth factors in place of cyanocobalamin, but methionine can replace the latter. This strain of B.coli responds equally well in a tube assay to cyanocobalamin, factors A, B or C, or pseudovitamin B_{12}. The author is much indebted to Dr. W. F. J. Cuthbertson of Glaxo Laboratories, Ltd. for supplying him with a culture of the M 200 variant of the C 181 strain of B.coli, and to Mr. G. E. Shaw of Evans Biological Institute for coming to Edinburgh to advise on the setting up of the B.coli assay technique./

technique.

Composition of medium for B.coli assay.

	Amount per 100 ml. of double strength medium.
NH_4 Cl	1 G.
Na_2 SO_4	400 mg.
KH_2 PO_4	200 mg.
NH_4 NO_3	200 mg.
K_2 H PO_4	600 mg.
Mg SO_4	20 mg.
Glucose	1 G.
Asparagine	300 mg.
*Mixed trace element solution	0.2 ml.
Ca Cl (1:1,000 solution)	0.1 ml.

Filter to clarify.

Then add stock iron solution** in a proportion of 0.1 ml. to 2 litres of double strength medium.

* Trace elements.

Stock solutions

Boron	5.7 G.	Boric acid in 1,000 ml. water
Copper	3.9 G.	Cu SO_4, $5H_2O$ " " " "
Manganese	4.1 G.	Mn SO_4, $4H_2O$ " " " "
Molybdenum	2.1 G.	Na MoO_4, $2H_2O$ " " "
Zinc	4.4 G.	Zn SO_4, $7H_2O$ " " " "

Mixed trace elements solution

Mix	630 ml.	Zinc	stock solution
	20 ml.	Molybdenum	" "
	40 ml.	Manganese	" "
	100 ml.	Copper	" "
	10 ml.	Boron	" "
	200 ml.	H_2O	

** Iron stock solution

1.0 G. Fe SO_4 $7H_2O$ in 25 ml. 1% HCl

This/

This is a modification of Shaw's medium and with cyanocobalamin it has given results comparable to those obtained with the L.leichmannii assay. Recovery experiments where cyanocobalamin had been added to urine, serum or nutrient broth gave satisfactory recoveries.

The medium for the B.coli assay does not require to have its pH adjusted. The procedure is in general similar to that for the L.leichmannii assay in that there are seven tubes in each row of standard, each containing a total volume of 10 ml. The standard cyanocobalamin is added so that the tubes contain respectively, 0, 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 m.µg. of the substance. This means that the assay is considerably less sensitive than that with L.leichmannii, and in addition the maximum turbidity that occurs is only about half that found with the L.leichmannii technique.

The standard cyanocobalamin or the test substance was autoclaved with the medium. An aseptic technique may, however, be used. The tubes were autoclaved for 10 minutes at 10 lbs., 10 minutes being taken for the pressure to rise and 10 minutes for cooling to occur.

Incubation was at 37°C. for 16-18 hours.

In some instances it was necessary to add a drop of 1% sodium cyanide to substances undergoing test with L.leichmannii or B.coli in order to convert vitamin B_{12b} or B_{12c} into B_{12}.

Streptococcus faecalis Assay for Folic Acid.

This is a modification of the method of Teply & Elvehjem (1945)./

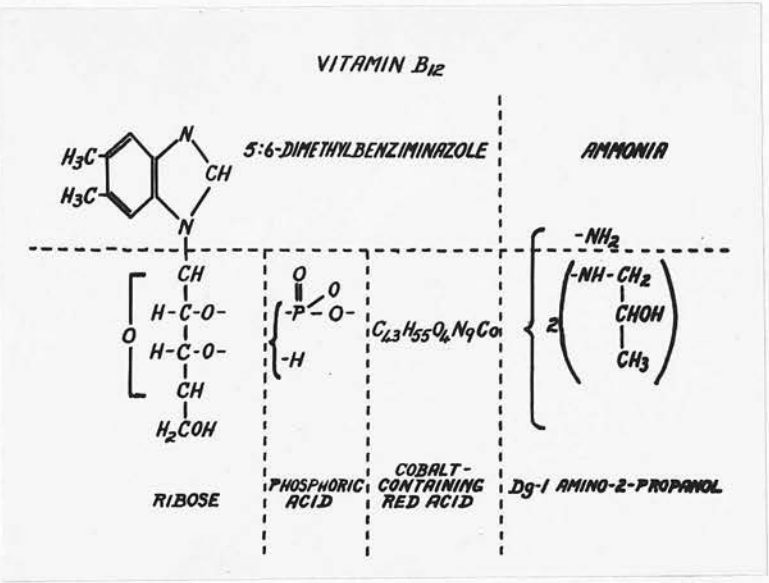
(1945).

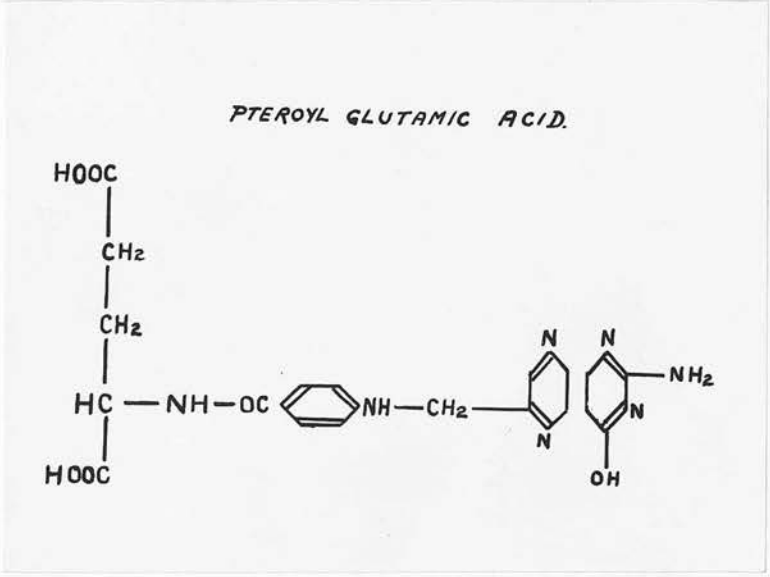
Constituent	Amount per 100 ml. of double strength medium
Casein, acid hydrolysed	1.0 G.
Glucose	4.0 G.
Sodium citrate	5.0 G.
Dl alanine	40 mg.
L-asparagine	20 mg.
L-cystine	40 mg.
Tryptophane	40 mg.
Adenine	2 mg.
Guanine	2 mg.
Xanthine	2 mg.
Uracil	2 mg.
p-aminobenzoic acid	2 µg.
Calcium pantothenate	80 µg.
Nicotinic acid	120 µg.
Pyridoxine hydrochloride	240 µg.
Riboflavin	40 µg.
Aneurin hydrochloride	40 µg.
Biotin	0.08 µg.
K_2HPO_4	0.5 G.
Salt B	1.0 ml.

The final pH was adjusted to 6.8. The standard was made up from synthetic pteroylglutamic acid powder and the standard tubes which, as in the other assays had a final volume of 10 ml., contained respectively 0, 1, 2, 4, 6, 8 and 10 mµg. of pteroylglutamic acid.

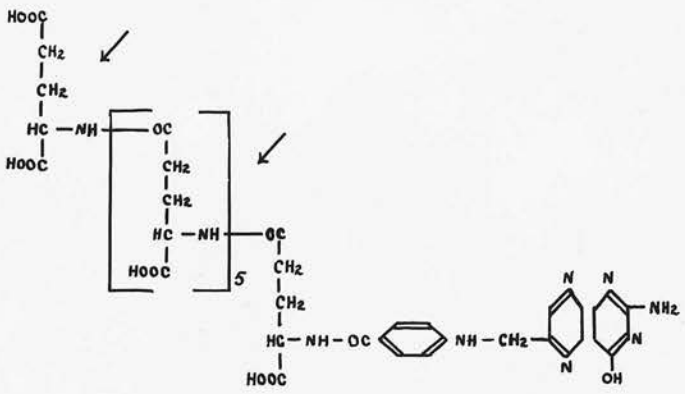
Apart from serum which was added aseptically, the medium was autoclaved with the standard or with the substance undergoing test. Autoclaving was for 10 minutes at 15 lbs. pressure and incubation after the inoculation with S.faecalis was for 16-18 hours at 37°C.

The test is not altogether specific for pteroylglutamic acid. Known substances that can replace pteroylglutamic acid under/

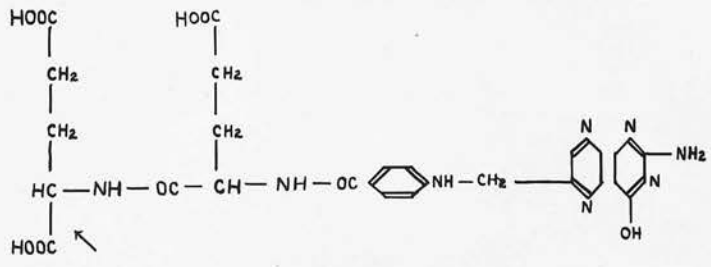

VITAMIN B_{12}
5:6-DIMETHYLBENZIMINAZOLE
AMMONIA
RIBOSE
PHOSPHORIC ACID
COBALT-CONTAINING RED ACID
Dg-1 AMINO-2-PROPANOL
$C_{63}H_{55}O_4N_9Co$


PTEROYL GLUTAMIC ACID.

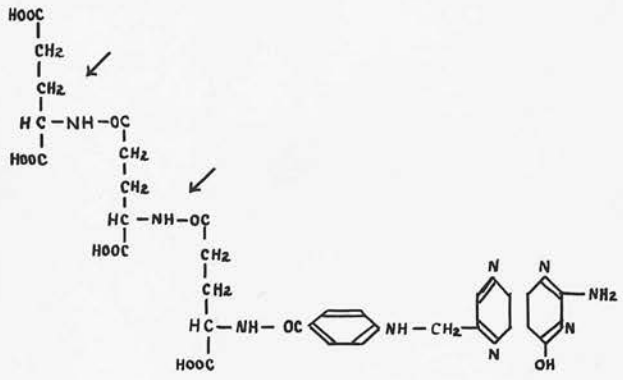
PTEROYL HEXAGLUTAMYLGLUTAMIC ACID



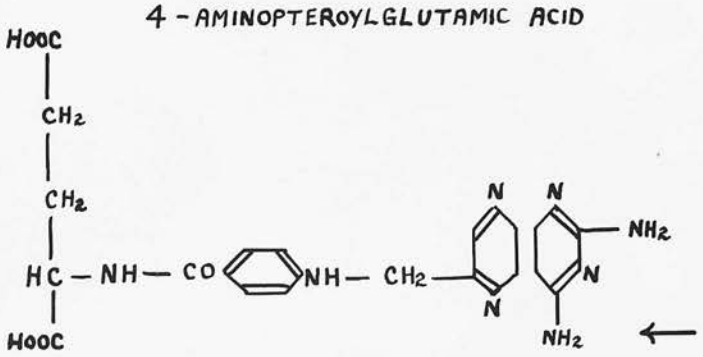
PTEROYL-ALPHA-GLUTAMYLGLUTAMIC ACID



PTEROYL-GAMMA-GLUTAMYL-GAMMA-GLUTAMYLGLUTAMIC ACID



4-AMINOPTEROYLGLUTAMIC ACID



under the conditions of the test are thymine, thymidine, citrovorum factor (5-formyl-5,6,7,8-tetrahydropteroylglutamic acid), N^{12}-formylpteroic acid which is found in the fermentation broth of Rhizopterin nigricans, pteroyl-γ-glutamyl-γ-glutamyl-glutamic acid (pteroyltriglutamic acid) which is found in the fermentation broth of Corynebacterium, pteroic acid which does not occur naturally, and the synthetic pteroyl-γ-glutamylglutamic acid. Substances which do not support the growth of S.faecalis are the synthetic pteroyl-α-glutamyl-glutamic acid (pteroyldiglutamic acid) and the naturally occurring pteroylhexaglutamyl glutamic acid.

There are no doubt other unidentified conjugates and analogues and for this reason it is better to use the term 'folic acid' except when pure pteroylglutamic acid is being considered. It is frequently necessary to correct the apparent folic acid content of a substance or solution for its content of citrovorum factor. Under such circumstances, the citrovorum factor may be assayed using Leuconostoc citrovorum as test organism and a citrovorum factor standard. An assay with Streptococcus faecalis using a pteroylglutamic acid standard will then give the apparent folic acid activity, but citrovorum factor content will be included in this reading. If a Streptococcus faecalis assay is carried out with two different standards, viz. pteroylglutamic acid and citrovorum factor, the results can be read in terms of pteroylglutamic acid or citrovorum factor. If the substance being tested contains/

contains only citrovorum factor, then the results will be the same with the citrovorum factor standard whether the test organism be L.citrovorum or S.faecalis. However, we have found citrovorum factor to have less activity for S.faecalis than has pteroylglutamic acid. Over a large series of experiments, the stimulation of growth of S.faecalis produced by 1.0 mg. of citrovorum factor (calculated as the free acid) was equivalent to that produced by 0.58-0.78 mg. of pteroylglutamic acid. There were variations with individual assays, but the results were consistently within this range. If, for example, we take an assay where the growth produced by 1.0 mg. of citrovorum factor was equivalent to that produced by 0.7 mg. of pteroylglutamic acid, we may say that each milligramme of citrovorum factor would falsely show itself in the S.faecalis assay as 0.7 mg. of pteroylglutamic acid.

The true pteroylglutamic acid content

= the folic acid activity (from the S.faecalis assay) minus $\frac{70}{100}$ x citrovorum factor content (from the L.citrovorum assay)

The extent of the growth stimulation of S.faecalis by citrovorum factor reported here is in keeping with the findings of Broquist (1951), but differs from the experience of Swendseid et al. (1951) who used folinic acid provided by Dr. William Shive as standard and found that 1.0 mg. of folinic acid had the growth-promoting activity of 1.5 mg. of pteroylglutamic acid.

Leuconostoc/

Leuconostoc citrovorum assay for Citrovorum Factor.

This was done by a modification of an unpublished method of Shive.

	Amount per 100 ml. of double strength medium
Acid hydrolysed casein	0.5 G.
l-asparagine	10 mg.
l-tryptophane	10 mg.
l-cystine	10 mg.
Sodium acetate	600 mg.
*Thymidine	10 µg.
Salt A	0.5 ml.
Salt B	0.5 ml.
Aneurin hydrochloride	20 µg.
Nicotinic acid	20 µg.
Calcium pantothenate	20 µg.
Riboflavin	20 µg.
Pyridoxine	340 µg.
Pyridoxal	100 µg.
Inositol	100 µg.
Biotin	0.2 µg.
p-aminobenzoic acid	1.0 µg.
Pteroylglutamic acid	1.0 µg.
Adenine sulphate	1.0 mg.
Guanine hydrochloride	1.0 mg.
Uracil	1.0 mg.
Xanthine	1.0 mg.
Glucose	1 G.

pH adjusted to 6.8

* This is not available commercially, but was supplied by the kindness of Dr. T. G. Brady of Dublin.

This is a relatively easy assay to perform. The standard and the substance undergoing test are autoclaved with the medium. The total volume is again 10 ml. The standard was supplied by Dr. H. P. Broquist of Lederle Laboratories Inc., Pearl River, New York, and to him the present author is much indebted. This standard was the calcium salt of citrovorum factor/

factor, 1 mg. of which is equivalent to 0.785 mg. of citrovorum factor. In the rows of standard, the tubes contained respectively 0, 0.5, 1, 2, 3, 4 and 5 mμg. of citrovorum factor, calculated as the free acid. Autoclaving was for 7 minutes at 15 lbs. pressure and after the tubes had been inoculated with L.citrovorum they were incubated at 37°C. for 16 hours. Turbidity was then read.

Maintenance of Organisms.

Lactobacillus leichmannii.

Daily transfers were made, inoculation being made into liquid medium and soya medium on alternate days.

Liquid medium. This consisted of assay medium No.1, diluted with equal amounts of water to give a single strength medium. To one litre of the single strength medium there was added 10 ml. of refined liver extract containing at least 12 μg. cyanocobalamin per ml.

Soya medium. Soya bean powder supplied by British Drug Houses, Ltd., was mixed with ten parts of distilled water till a satisfactory cream was formed. This was sterilised by being steamed for half an hour on three successive days.

When growth took place in this medium, clotting of the medium occurred.

Bacillus coli.

The strain was carried on nutrient agar slopes subcultured every 7 days, incubated for 24 hours and stored at 4°C.

Nutrient/

Nutrient Agar.

1 lb. minced, trimmed veal
1 litre tap water

Leave overnight at room temperature. Boil 15 minutes and filter through muslin. Add to the filtrate peptone (1 G. per 100 ml.) and sodium chloride (0.5 G. per 100 ml.). Adjust pH to 7.6 Heat to 85°C. for 5 minutes and filter. Add Davies' Agar (1 G. per 100 ml.). Boil to dissolve.

Distribute in tubes, autoclave at 10 lbs. for 10 minutes and allow to cool in the sloping position.

Streptococcus faecalis.

The organisms were transferred daily, liquid and solid media being used on alternate days.

The liquid transfer medium was the same as the assay medium, diluted with equal parts of water to give single strength medium, and to it there had been added a refined liver extract (Anahaemin) in a volume of 20 ml. per litre of single strength medium.

The solid medium was an agar stab.

Agar medium.

Yeast (baker's)	1 G.
Glucose	1 G.
Agar	1.5 G.
Water to	100 ml.

The medium was made up in the above proportions, steamed at 100°C. for 2 hours and filtered with the aid of a vacuum pump.

Approximately 10 ml. portions of the hot solution were poured into test tubes, which were plugged with non-absorbent cotton wool and sterilised by steaming for

30/

30 minutes at 100°C. on three successive days. The medium was allowed to solidify in upright position.

Leuconostoc citrovorum.

This organism was also subcultured daily, liquid medium and soya medium being used on alternate days. The media used were the same as these used for maintaining L.leichmannii.

The Inoculum.

The same procedure was adopted with L.leichmannii, S.faecalis and L.citrovorum. Cultures growing in liquid transfer medium were taken and all but an approximate 0.1 ml. was decanted off. This residue was suspended in 5 ml. of normal saline. An approximate 4.5 ml. of this was then decanted off and the volume again made up to 5 ml. with normal saline. This was used for the inoculum, one small drop being used.

With B.coli the suspension for inoculum was made either by washing off a nutrient agar slope which was at least 7 days old, or by subculturing from an agar slope into starter broth, incubating overnight and subculturing this into fresh starter broth, using a heavy inoculum and incubating for six hours. (The starter broth was made by adding 20 μg. of cyanocobalamin to 1 litre of single strength medium, distributing in 10 ml. quantities, and autoclaving at 10 lbs. for 10 minutes.) In each case the suspension was washed twice with assay medium before final suspension in assay medium. The inoculum was of the order of opacity 1/5 B.W. opacity tubes.

ASSAY/

The following results of triplicate assays have been taken completely at random from our records, to illustrate the degree of accuracy that occurs with microbiological techniques.

Urinary folic acid excretion after a test dose of PGA (mg.).

				Mean.
Case 1	2.99	2.84	2.75	2.86
2	2.73	2.68	2.57	2.66
3	2.60	2.59	2.25	2.48
4	2.54	2.41	2.00	2.32
5	2.43	2.34	2.04	2.27
6	2.32	2.22	2.19	2.24
7	1.91	1.88	1.85	1.88
8	1.85	1.77	1.74	1.79
9	1.004	0.965	0.928	0.966
10	0.213	0.210	0.183	0.202

Urinary citrovorum factor excretion after a test dose of PGA (µg.)

				Mean
Case 1	56.0	45.0	53.2	51.4
2	23.7	24.8	22.5	23.7
3	15.7	18.9	15.6	16.7
4	12.5	10.4	11.2	11.4
5	9.0	11.9	7.2	9.3
6	5.1	5.5	6.3	5.6
7	3.0	2.7	2.8	2.8
8	2.5	2.6	2.5	2.5
9	0.76	0.76	0.74	0.75
10	0.57	0.43	0.72	0.57

So far as vitamin B_{12} is concerned, the results of triplicate assays on specimens of serum are given on page 312.

Various recovery experiments are described in the relevant chapters.

PREPARATION OF TISSUES FOR ASSAY OF THEIR CONTENT OF HAEMOPOIETIC FACTORS.

Two methods were employed.

Method A. Approximately 1 G. of tissue was homogenised in 10 ml. of 0.2 M-phosphate buffer solution at pH 7.5. There were added a further 10 ml. of the same buffer solution and the sample was incubated at 37° C. for 24 hr. The material was then steamed for 10 minutes at 100° C. and filtered. The volume was made up to 50 ml., the final pH being adjusted to 6.8 with 0.1 N-HCl.

Method B. To approximately 1 G. of tissue there were added 20 mg. of pancreatin which itself was found to have negligible growth-promoting activity for the test organism. Then 10 ml. of 0.2 M-phosphate buffer at pH 6.8 were added and the further steps carried out as in method A, the buffer used throughout being of pH 6.8. The final pH was 6.8.

Buffer solution.

Stock solutions used for the preparation of buffer solutions were (i) 27.228 G. KH_2PO_4 dissolved in distilled water and made up to 1 litre. (ii) 35.612 G. $Na_2HPO_4.2H_2O$ dissolved in distilled water and made up to 1 litre. 0.2 M-phosphate buffer solution at pH 7.5 was prepared by mixing 84.2 ml. of solution (i) with 15.8 ml. of solution (ii). 0.2 M-phosphate buffer solution at pH 6.8 was prepared by mixing equal parts of solutions (i) and (ii).

All pH estimations were made with glass electrodes.

SUMMARY.

In this chapter there has been given a brief description of the basic techniques that were finally evolved for the carrying/

carrying out of the investigations described in the pages that follow. Certain more specialised techniques will be mentioned in the relevant chapters.

INVESTIGATIONS OF THE INTESTINAL CONTENT OF GROWTH FACTORS FOR L.LEICHMANNII AND S.FAECALIS, OF THE ABILITY OF GASTRO-INTESTINAL BACTERIA TO SYNTHESISE OR DESTROY HAEMOPOIETIC FACTORS, AND OF THE INFLUENCE ON THIS OF NORMAL GASTRIC JUICE

In 1928 Davidson showed that in pernicious anaemia there is an abundant bacterial flora in the stomach and duodenum, but thereafter the investigations of Castle and his co-workers, which seemed to explain the pathogenesis of pernicious anaemia, caused most workers in this field to forget about the bacteriology of the alimentary tract in pernicious anaemia, and associated conditions. Interest in this aspect of the subject has revived in recent years.

In 1948 Bethell and his co-workers showed that the faeces of a pernicious anaemia patient had microbiological vitamin B12 activity, presumably because of synthesis by intestinal bacteria, and Bethell (1948) then showed that an extract of the stools produced a haemopoietic response if given by injection to another pernicious anaemia patient.

For this reason the present author, when working at Ann Arbor in 1948, thought it would be of interest to pass an intestinal tube as far down the alimentary tract as possible in fasting pernicious anaemia patients and in controls, and to assay the intestinal contents for what were then considered to be vitamin B12 and pteroylglutamic acid. The main object was to/

to have this information as a pilot study for a more extensive investigation of the extent to which spontaneous remissions and relapses in pernicious anaemia might be due to bacterial synthesis or destruction of haemopoietic factors. Simultaneously the work on the effect of succinylsulphathiazole on the production of anaemia in guinea-pigs was being done as described in Chapter 3. On the completion of the pilot study this type of research was temporarily suspended, partly because of the negative results of the initial investigations and partly because it became evident that the assays were not measuring only vitamin B_{12} and folic acid.

The next phase in the present series of investigations, apart from a few unsuccessful attempts to treat pernicious anaemia patients with penicillin, was conducted in 1952 with the assistance of Dr. J. Somner, who isolated various strains of B.coli from the contents of the stomach or small intestine of patients with pernicious anaemia and a few other conditions. Attempts were made to show that these organisms produced or "destroyed" cyanocobalamin, pteroylglutamic acid or citrovorum factor, when the organisms were incubated with small quantities of these haemopoietic factors. As will be seen, the experiments gave largely negative results. Dr. Somner discontinued her experiments on the isolation of bacteria in March 1953 and thereafter a few further investigations with mixed flora from the alimentary tract of pernicious anaemia patients also gave essentially negative results.

In/

In October 1953 the experiments were extended to include measurement of the possible effects of the alimentary bacteria on larger amounts of cyanocobalamin, and, as is described later in this chapter, different media were employed and the technique was altered slightly. It was now found possible to demonstrate that certain organisms from the stomach or small intestine of pernicious anaemia patients would remove cyanocobalamin when incubated with it in a suitable medium.

When cyanocobalamin was isolated in 1948 and shown to be allied to both the 'extrinsic factor' and the 'specific anti-anaemic factor', two possible mechanisms required consideration. One was that the intrinsic factor combined in some way with cyanocobalamin and promoted its absorption, and the alternative was that normal gastric juice prevented the destruction or inactivation of cyanocobalamin by intestinal bacteria. For this reason one of the subsidiary considerations in the experiments done at Ann Arbor was if possible to collect evidence for or against this second theory. As will be seen the results were not conclusive.

It was obvious in 1953, when it was found that certain bacteria were capable of removing cyanocobalamin from a suitable culture medium, that the next step was to see whether or not normal gastric juice prevented this action. Meantime at least two other investigators were working along similar lines (Burkholder, 1952; Hoff-Jorgensen et al., 1952).

The more important relevant features in the literature are as follows:

Mode/

Mode of Action of Intrinsic Factor.

In 1949 Ternberg & Eakin showed that normal gastric juice and gastric tissues contained a "vitamin B_{12} binding protein" which they named apoerythein. This protein prevented the utilisation of vitamin B_{12} by organisms which required vitamin B_{12} for growth. Ternberg & Eakin then claimed, for no very obvious reason, that apoerythein was intrinsic factor. The gastric juice of pernicious anaemia patients contained reduced amounts of this protein, but Bird & Hoevet (1951) have shown that many substances can 'bind' vitamin B_{12}, so that although the observations of Ternberg & Eakin were of much interest, they were not proof of the fact.that the vitamin B_{12} binding substance was intrinsic factor. This objection is being raised at once because the same difficulty applies to our own experiments wherein attempts were made to see whether various samples of gastric juice prevented the uptake or destruction of cyanocobalamin by strains of B.coli which did not themselves require cyanocobalamin for growth (see p.239). The fact that pernicious anaemia gastric juice does not prevent such uptake or destruction does not prove that it is intrinsic factor in normal gastric juice that does. Even if a gastric juice or gastric preparation which prevents suitable strains of B.coli from removing cyanocobalamin from a culture medium promotes a haemopoietic response when given orally with cyanocobalamin to an untreated pernicious anaemia patient, this still does not prove that it is intrinsic factor that is having the effect on the/

the B.coli.

Moreover if strains of B.coli or other organisms isolated from the stomach or jejunum of pernicious anaemia patients are capable of destroying or absorbing cyanocobalamin this does not prove that this mechanism is of primary importance in the body. If, on the other hand, such organisms are capable of producing cyanocobalamin and we accept the alternative explanation that intrinsic factor combines with cyanocobalamin and thereby promotes its absorption, it follows that the pernicious anaemia patient is not likely to benefit from this activity of the micro-organisms since he will not be able to absorb the vitamin B_{12} that is produced.

It may be said that there is little direct evidence to support any hypothesis in connection with the mode of action of intrinsic factor.

Ungley (1950) carried out a series of experiments which included (i) the instillation of cyanocobalamin with or without gastric juice into a washed loop of intestine between two balloons of a Miller Abbott tube in two patients, and (ii) the giving to another case of aureomycin and other drugs until the stools were sterile, 80μg. of cyanocobalamin thereafter being administered by mouth. No response occurred in any of these cases. This latter experiment was the more convincing one, especially since the patient then responded when 80 μg. of cyanocobalamin was given by mouth with normal gastric juice. If the function of intrinsic factor is to prevent intestinal bacteria from destroying cyanocobalamin, one would expect a pernicious anaemia patient with a presumably sterile small intestine/

intestine to respond to 80 μg. of cyanocobalamin by mouth. On the other hand Lichtman et al. (1950) found that in pernicious anaemia the daily oral administration of 3 μg. of cyanocobalamin was ineffective until aureomycin was given: a good response then occurred. Against this, however, Watson (1951) has reported small and delayed responses in pernicious anaemia to aureomycin alone.

Welch et al. (1952) have found that if oral doses of cyanocobalamin containing radioactive cobalt are given to pernicious anaemia patients, the faecal excretion of radioactive cobalt usually exceeds 70% unless a source of intrinsic factor is given simultaneously, when it is reduced to 25% or less. Similar results were reported by Witts' group at the Association of Physicians meeting in 1953. This again is not absolute proof that intrinsic factor combines with cyanocobalamin since there remains the possibility that in the pernicious anaemia patients intestinal micro-organisms absorbed the radioactive cyanocobalamin and carried it out in the stools, while the source of intrinsic factor, when given to the patients by mouth, prevented this action so that the radioactive cyanocobalamin was absorbed to a greater extent.

In brief, therefore, we have no direct knowledge of the mode of action of intrinsic factor on vitamin B_{12}.

Ability of Microorganisms to produce or "destroy" Haemopoietic Factor.

Many workers have published papers on this subject, and it has been found that different strains of the same organism may differ/

differ in their ability in this respect. When a haemopoietic factor seems to disappear from a culture medium in which an organism is growing, this may be because it is destroyed, conjugated or modified in some way or because it is absorbed by the organism, or perhaps because there is produced some other substance which interferes with the test for the haemopoietic factor that is under consideration. Moreover, what happens between a certain strain of organism and a haemopoietic factor under one set of circumstances may be different from what happens if the environmental conditions are changed. Differences in temperature, or in the culture medium used or the presence of other organisms may be of importance in this respect.

Papers dealing with the ability of bacteria to produce or eliminate pteroylglutamic acid have been written by Hutchings et al. (1941), Mitchell & Isbell (1942), Thompson (1942) and numerous others.

Some of the organisms that have been tested for their ability to produce folic acid or remove it from a culture medium are shown in Table 23. (Various workers)

It will be seen that, for example, different strains of S.faecalis may produce folic acid, remove it, or do neither.

So far as cyanocobalamin is concerned, the position is extremely confused in that it is only very recently that it has been realised that bacteria may produce a variety of substances with cyanocobalamin like activity for certain test organisms, especially for the mutant of B.coli used in vitamin B_{12} assays.

For/

TABLE 23.

ORGANISMS KNOWN TO PRODUCE OR UTILISE FOLIC ACID OR TO DO NEITHER

Folic acid produced	Folic acid removed from a medium	Neither
S.faecalis (3 strains known to do this)	Strep.lactis R	L.arabinosus 17-5
Aerobacter aerogenes (aerobic)	Strep.faecalis 732	Leuconostoc mesenteroides 6205
Aerobacter aerogenes (anaerobic)	Strep.faecalis F24	
Serratia marcesans	Strep.zymogenes 5C1	Strep.lactis 374, 4487, 8039, 7963, 4386, L103, L104, L206
Pseudomonas fluorescens	Strep.durans 98A	
Proteus vulgaris	L.casei	
Clostridium butylicum	L.delbruckii LD5	Strep.faecalis 10C1
Bacillus lactis acidi	L.bulgaricus 05	Strep.zymogenes 6054
Lactobacillus pentosus	Strep.casei 19	
Bacillus brassicae	Strep.faecalis S108A	
Leuconostoc mesenteroides	Clostridium tetani	
Lactobacillus gayonii	L.helveticus	
	Propionibacterium pentosaceum	

For this reason we do not know what some of the investigators were measuring. Halbrook (1952) using a L.leichmannii method of assay, stated that practically all micro-organisms isolated from poultry litter and droppings, including bacteria, yeasts and moulds produced some vitamin B_{12} in the medium in which they were grown. He isolated 142 organisms. In 1952 Davis et al. allowed several strains of micro-organisms to grow in a basal synthetic medium to which radioactive cyanocobalamin tagged with Co^{60} was introduced. The organisms chosen were L.leichmannii, L.arabinosus, B.coli, S.lactis R, B.subtilis, P.mirabilis, B.mycoides, Esch.freundii, S.dobson and L.lactis Dorner. Of these, radioactivity was found in varying amounts in the cells of all species except L.arabinosus. The incorporation of the radioactivity was not related to the requirements of cyanocobalamin for growth of the bacterial cells. It appeared likely that the radioactivity in the bacterial cells was due to vitamin B_{12}, because cells grown in the presence of Co^{60} Cl_2 with or without addition of non-radioactive cyanocobalamin contained no radioactivity and the microbiological activity in homogenates of disintegrated cells paralleled that calculated from radioactivity due to Co^{60}.

Clinical experiments on the action of antibiotics and sulphonamides in the treatment of megaloblastic anaemia.

Mention has already been made of the experiments of Ungley (1950) who had no response in one case of pernicious anaemia to orally administered cyanocobalamin after the administration of aureomycin, streptomycin and phthalylsulphathiazole/

phthalylsulphathiazole. Lichtman et al. (1950) had a response to 3 μg. of cyanocobalamin by mouth when aureomycin was given. Davis (1951) had no response to aureomycin alone, but a good response to cyanocobalamin together with aureomycin. In other cases, however, he had no results (1952).

From Africa, Foy & Kondi have published a series of papers dealing with the haematological effects of antibiotics in patients with various types of megaloblastic anaemia. (Foy et al. 1951a,b,c, 1952a,b). They tell of a negress who had megaloblastic anaemia which may have been pernicious anaemia and who responded to injections of 400,000 units of crystalline penicillin daily, and of another with megaloblastic anaemia of pregnancy who showed a good response to treatment with the same substance in a similar dosage. In these two cases, provided coincidental spontaneous remission is excluded, penicillin must either have had a haemopoietic effect or it must have been excreted into the alimentary tract where it might theoretically stimulate bacteria that produce haemopoietic factors, inhibit those that "destroy" them, or inhibit bacteria that produce haemopoietic antagonists. Alternatively it might destroy factors interfering with absorption or utilisation of haemopoietic factors or have some effect on protein metabolism and the synthesis of nucleo proteins from basic amino acids (Foy et al., 1951b).

A later paper (1952a) published by these workers is of less value because although they treated successfully a patient who had megaloblastic anaemia of pregnancy with an animal/

animal protein factor preparation, it contained not only aureomycin but also vitamin B_{12}. The most recent conclusion of these workers is that doses of less than 200,000 units daily by mouth or 400,000 units by injection cause haematological improvement, whereas larger doses do not, and that this suggests that penicillin produces its effects by changing the intestinal microflora. Some of our own experiences with penicillin in pernicious anaemia are recorded later (p.424,441).

That antibiotics can cause alteration in the normal flora of the body is a matter about which there can be no doubt. For instance Long (1947) and Cross & Boots (1948) showed that a few days after the injection of penicillin, coliform bacilli appeared in the throat, the normal Gram positive population changing to a Gram negative one. Oleson et al. (1950) showed that when chicks were fed a diet deficient in animal protein factor they did not respond as regards growth to aureomycin unless there was vitamin B_{12} in the diet. There seemed to be a mutual sparing action between vitamin B_{12} and aureomycin. Biely & March (1951) found that chicks on a folic acid deficient diet did not gain weight and were poorly feathered. If given aureomycin they did gain weight and feathering was normal. Moreover aureomycin could compensate for dietary insufficiency of nicotinic acid or riboflavin, and it seemed at least possible that the aureomycin was reducing the number of intestinal microflora that might compete with the host for members of the vitamin B complex.

Our own experiments with guinea-pigs as described in Chapter/

Chapter 3 have some bearing on this matter: at least they show something of the complexity of the problem of what sulphonamides and antibiotics do in relation to haemopoiesis. It is of interest that Waisman (1952) found that aminopterin added to the diet of the rat caused anaemia and impairment of growth. In the rat this could be prevented by large doses of folic acid or small doses of citrovorum factor. Aureomycin whether given by mouth or by injection could also prevent the deleterious effect of aminopterin. However aureomycin would not overcome the growth inhibiting effect of phthalylsulphathiazole. All this reinforces the view that we cannot at present be certain whether aureomycin acts by its effect on intestinal bacteria, by an effect on tissue metabolism, or both.

It is perhaps pertinent to mention at this point that Gale & Taylor (1946) showed that the effect of penicillin on Staph.aureus is to prevent the assimilation of glutamic acid. There occurs a decrease of the internal concentration of glutamic acid in the bacterial cells, and cessation of growth occurs. In 1947 the same workers, using S.faecalis cells showed that the effect of the antibiotic tyrocidin on them was to cause lysis and leakage of the internal amino acids. Loomis et al. (1948) showed that mepacrine inhibited oxidative phosphorylations and probably it has this effect on bacteria as well as tissue cells. Cross et al. (1949) showed that gramicidin had a similar effect and Loomis (1950) claimed that aureomycin also does this. However van Metier & Oleson (1951) prefer to believe that aureomycin blocks some part of the Krebs cycle.

Substances/

Substances occurring in the alimentary tract that simulate Cyanocobalamin in Microbiological Assays.

As will be described in the pages that follow, an attempt was made by the present author when at Ann Arbor to measure the concentration of haemopoietic factors in the intestinal contents and the stools of several patients.

In the light of the knowledge available at that time it was thought that the S.faecalis assay was measuring pteroylglutamic acid and that the L.leichmannii assay was measuring vitamin B_{12}. Unfortunately the matter is much more complex than that. We have already seen (p.180) that the S.faecalis assay measures citrovorum factor (and perhaps citrovorum factor conjugates) in addition to pteroylglutamic acid; so far as the contents of the intestinal tract are concerned, at least five substances are being measured in the assay for vitamin B_{12} despite the use of a technique that includes alkaline hydrolysis. Fortunately this complication does not arise in the measurement of the amount of vitamin B_{12} in the liver. (Ford et al., 1953b).

The five substances under consideration are now known in Britain as factors A (vitamin B_{12m}), B and C, cyanocobalamin and pseudovitamin B_{12}. (Ford, 1953; Holdsworth, 1953; Ford et al., 1953a). The other substances are quite distinct from the vitamin B_{12} compounds and their existence was suspected from finding that various test organisms used in assaying vitamin B_{12} gave very different results when attempts were made to measure the quantities of vitamin B_{12} in the faeces of animals. The substances are separable by complicated studies involving/

involving chromatography and ionophoresis, the chief difficulty being that their presence has to be shown by bacteriological methods as they are not visible. Quite apart from this great complication, if the extraction of faeces is not done in the presence of cyanide, other cobalamins such as hydroxocobalamin and nitritocobalamin may not be measured in the assay, and if a technique that includes alkaline hydrolysis is not done in the L.leichmannii assay, substances such the desoxyribosides may be falsely measured as vitamin B_{12}.

The haemopoietic activity of the 'new' factors A, B, C and pseudovitamin B_{12} have not yet been fully established, but factor A was said to be as active as cyanocobalamin when given by injection to a single case of pernicious anaemia. (Coates et al., 1952). More recently Potter (1953), one of the same group of workers, has cast doubts on the freedom from vitamin B_{12} of the substance they were testing.

Unfortunately, not only is the growth ratio of these various factors different for each of the vitamin B_{12} test organisms, but the ratio varies according to the conditions of the test, even with the same organism. It is perhaps best to illustrate this by a table prepared from information kindly supplied by Ford prior to its publication. (Table 24.)

This table indicates that the most specific method of assay is the new one involving the protozoon Ochromonas malhemensis. (Ford, personal communication.) At the time of writing nothing has been published about this method of assay, and it may be that further factors will be discovered by this new technique.

The/

TABLE 24.

Relative Vitamin B_{12} Activities of different Compounds, measured by different Assay Techniques (Ford).

Results are expressed in terms of the activities measured by the B.coli tube assay given as 100 in each instance.

Substance	B.coli (tube) method	B.coli (cup-plate method)	L.leichmannii	Euglena gracilis	Ochromonas Malhemensis
Cyanocobalamin	100	100	100	100	100
Factor A	100	274	64	137	3.4
Factor B	100	580	< 0033	4.2	0
Factor C	100	8500	14	100	0
Pseudo-vitamin B_{12}	100	1000	400	800	1.2

The L.leichmannii assay measures, in particular, cyanocobalamin, factor A and pseudovitamin B_{12}. Ford (1952) claims that factor A is the most abundant active compound in cyanide extracts of calf faeces, but if it is true that the substance has haemopoietic activity in pernicious anaemia, the L.leichmannii assay would perhaps remain the one of choice in investigations performed on faeces or samples of intestinal contents. It remains to be seen whether or not intrinsic factor is required for the absorption of factor A. The clinical activity of pseudovitamin B_{12} has been investigated.by Pfiffner and his colleagues who in 1951 described the isolation of six Co-containing pigments closely related to vitamin B_{12}. They were produced under anaerobic conditions by an incompletely identified organism obtained from bovine rumen contents, and two were isolated in crystalline form. These workers claimed that pseudovitamin B_{12} is not active in pernicious anaemia, and so the next piece of information required concerns the extent to which "vitamin B_{12}" as assayed in the faeces, intestinal secretions or culture medium with L.leichmannii, is in fact pseudovitamin B_{12}. Such information is not yet available.

The crystalline compounds isolated by Pfiffner consisted of cyano- and hydroxo- forms (pseudovitamin B_{12b}), and in later studies (1953) the same investigators reported that the two crystalline 'pseudo' compounds contain adenylic acid in place of the phosphorylated riboside of 5,6-dimethylbenzimidazole. It is suggested that these compounds are intermediates in/

in the biosynthesis of cyanocobalamin and that it is therefore possible that the vitamin B_{12} requiring lactobacilli can replace the adenylic acid by the acquisition by the molecule of the biological activity characteristic of vitamin B_{12}.

In view of the known existence of the above substances and our inadequate knowledge of their characteristics, any conclusions about the content of haemopoietic factors in the intestinal contents, the stools or in artificial culture media must be treated with great reserve. In the light of present knowledge it is not possible to say whether the most satisfactory test organism is Ochromonas which is undergoing investigation, or L.leichmannii. Fortunately these four 'new' factors do not appear to occur to any extent in the liver or indeed anywhere other than in environments where bacterial growth is occurring, so that the L.leichmannii assay still has a definite place in investigative work.

SOME OBSERVATIONS ON THE INTESTINAL CONTENT OF FACTORS FOR THE GROWTH OF STREPTOCOCCUS FAECALIS AND LACTOBACILLUS LEICHMANNII

This work was carried out at the University of Michigan, Ann Arbor, Michigan in 1948-1949.

OBSERVATIONS ON PATIENTS.

Investigations were carried out on three patients suffering from pernicious anaemia, and two controls, one with rheumatoid arthritis, and the other with obesity. The intention was to measure the amounts of pteroylglutamic acid and vitamin B_{12} in the gastrointestinal secretion of these patients at various levels from the stomach, going down, if possible to the caecum. It is now known that the folic acid assay would include citrovorum factor and that the vitamin B_{12} included any factor A and perhaps pseudovitamin B_{12}. A sterile Cantor tube was passed, and the position of the tube was checked at intervals by means of fluoroscopy. No food or drink was given during the period of the investigations, which lasted for some thirty-six hours. Specimens were withdrawn with a sterile syringe. A specimen of stool was obtained at the termination of the experiment. In all cases, the tube appeared to reach the terminal ileum, but bacterial counts indicated that the caecum itself was not entered, although fluoroscopy suggested that it had been reached in Cases 1 and 2.

METHOD.

The folic acid assay was done with S.faecalis as described

in/

in Chapter 4. The 'vitamin B_{12} assay' was by Method 1, described in the same chapter. Sodium cyanide was not added as this modification was not known at the time, and thioglycollic acid was not used in this part of the investigations since under the conditions of the assay as performed at Ann Arbor it merely gave high blanks without increasing the amount of vitamin B12 measured by the assay. Enzyme preparations were not added to the stools or contents of the alimentary canal. The extract of the stools was a watery one, made by extracting with water and filtering through filter paper. Sterilisation was performed by autoclaving in the course of the assay.

The fasting gastric contents of both control patients contained free hydrochloric acid; the patients suffering from pernicious anaemia had histamine fast achlorhydria and a megaloblastic marrow, and were in every way typical cases of untreated Addisonian pernicious anaemia. The control patient suffering from obesity had been taking a low calorie diet for some time prior to the present investigation. The other patient had been eating a normal diet, but the patients with pernicious anaemia had perhaps eaten less because of lack of appetite.

Bacterial counts were carried out on each sample, various dilutions of the sample being plated on Brain Heart Infusion Agar, and counts being made at the end of a three day period of incubation. The specimens were not cultured separately for anaerobes./

anaerobes. Since all the tests on each specimen could not be carried out immediately, some samples were kept frozen in a deep freeze cabinet, but this did not prove satisfactory and the counts carried out on frozen samples are not included in our results. The faecal specimens were dried slowly at a low temperature. For bacterial counts, undried specimens were used, and a correction then made for the dried weight. The control patients had normal blood counts. At the time of the investigation the counts of the other patients were: Case 1 haemoglobin 6.7 G./100 ml., red blood cells 1,600,000/c.mm. Case 2 haemoglobin 12.1 G./100 ml., red blood cells 3,700,000/c.mm. Case 3 haemoglobin 4.2 G./100 ml., red blood cells 1,700,000/c.mm.

RESULTS OBTAINED.

The results of the investigation are given in Table 25. It should be remembered that the marking that has been reached on the intestinal tube may give little indication of the distance that the tube has passed along the intestinal tract. In all five cases, fluoroscopy indicated that the last specimen was withdrawn at least from the terminal region of the ileum.

In one patient with severe ulcerative colitis in whom an attempt had been made to pass a long intestinal tube to the caecum in order that the caecal contents could be aspirated continuously, a specimen of the fluid thus withdrawn was tested for L.leichmannii and S.faecalis factors. The results of/

TABLE 25

Content of Growth Factors for L.leichmannii and S.faecalis at various levels of the Gastrointestinal Tract.

Case	Diagnosis	Specimen	Growth factors for L.leichmannii (as vitamin B_{12} (µg./ ml.)	Growth factors for S.faecalis (as pteroyl-glutamic acid) (µg./ ml.)	Bacterial Count (Colonies per ml.)
1	Pernicious anaemia	Gastric juice	.005	.076	
		Duodenal	.0065	.042	
		Jejunal	.0057	.038	
		4 feet	.0053	.077	
		5 "	.0068	.183	
		5½ "	.0054	.46	
		5¾ "	.0040	-	
		6½ "	.0048	.336	
		Faeces	.1112 (per G. dried)	10.0 (per G. dried)	520,000,000 (per G. dried)
2	Pernicious anaemia	Gastric juice	.0076	.013	
		Duodenal	.0013	.014	
		5¾ feet	.00194	.24	
		6½ "	.00258	.65	
		Faeces	.1435 (per G. dried)	14.0 (per G. dried)	380,000,000 (per G. dried)
3	Pernicious anaemia	Gastric juice	.0019	.125	480,000
		Duodenal	.0014	.055	Negligible
		4 feet	.00166	.047	Negligible
		4½ "	.00216	.113	Negligible
		5 "	.0046	.115	100
		5½ "	.0059	.225	Negligible
		Faeces	.125 (per G. dried)	12.2 (per G. dried)	410,000,000 (per G. dried
4	Rheumatoid arthritis	Gastric juice	.0083	.039	Negligible
		Duodenal	.0012	.083	Negligible
		4 feet	.0087	.010	120
		5 "	.00103	.010	Negligible
		5½ "	.0015	.080	Negligible
		7 "	.0020	.068	Negligible
		7½ "	.0021	.083	180
		Faeces	.052 (per G. dried)	9.4 (per G. dried)	15,200,000 (per G. dried)
5	Obesity	Gastric juice	.00031	.038	3,200
		Duodenal	.00065	.020	240
		Jejunal	.00115	.011	1,450,000
		6½ feet	.00066	.013	52,000
		7 "	.0021	.013	70,000
		8 "	.0008	.018	4,700,000
		8½ "	.0004	.050	1,960,000,000
		Faeces	.126 (per G. dried)	14.8 (per G. dried)	4,900,000 (per G. dried)

of these tests were .0305 microgrammes per ml. of "vitamin B_{12}" and 12.1 microgrammes per ml. of "folic acid". Using the medium referred to above, colony counts revealed 1,200,000 colonies per ml. Although the material withdrawn was faecal, fluoroscopy revealed that the tube had not quite reached the caecum. At the time that this specimen was obtained the patient was receiving only fluids by mouth.

BACTERIOLOGY.

Since this was only a pilot investigation carried out simultaneously with a rather extensive animal investigation by a single worker with no technical assistants to do bacteriology, haematology, animal feeding or even cleaning of glassware, no effort was made to carry out detailed bacteriological studies on the intestinal secretions of most of these patients, but the specimens were studied microscopically, Gram's stain being used. No attempts were made to grow anaerobic organisms from most of the samples, but Moench et al. (1925), Davidson (1928) and other workers have shown an increase of intestinal flora, including B.welchii at abnormally high levels of the intestinal tract. In the present series, the organisms seen in the gastric and duodenal contents were mainly Gram negative bacilli and Gram positive cocci. The chief organisms present in the lower part of the small intestine of both the pernicious anaemia patients and the controls were Gram negative bacilli.

It will be seen in Table 25 that Case 5 who had a large number of organisms in the small intestine had the smallest amount of both the L.leichmannii and S.faecalis factors, but

that the content of these factors was similar in the stomach to what it was in the lower end of the ileum. The predominant organisms present in the terminal part of this patient's ileum were identified as Aerobacter cloacae. We have already seen that the patient with ulcerative colitis had high values for S.faecalis and L.leichmannii growth factors in the contents of the terminal ileum. The predominant organisms in the material aspirated were Pseudomonas pyocyaneus, Streptococcus viridans (mitis) and Clostridium innominatum.

DISCUSSION.

It will be seen that the maximum content of vitamin B_{12} in the upper intestinal tract (if indeed, this is the only factor being measured by this test) is.0065 microgrammes per ml. in Case 1. The very small amount that this represents may be gauged by the fact that Hall et al. (1949) had no response to the oral administration of 5 microgrammes of vitamin B_{12} alone daily in pernicious anaemia, although Dameshek (1950) has claimed a response with this dosage. Hall (reported by Bethell et al., 1949) had a slight response to 150 microgrammes given orally without a source of intrinsic factor, and West & Reisner (1949) had a definite response to 600 microgrammes given by mouth.

If conclusions may be based on variations in such small quantities, these observations may be considered as evidence that, in fasting pernicious anaemia patients, the content of growth factors for L.leichmannii in the small intestine is neither less nor more than in control subjects.

So/

So far as growth factors for S.faecalis are concerned there was rather more in the gastrointestinal secretions of the pernicious anaemia patients than in the controls, but the maximum found was only 0.65 µg. per ml. in Case 2.

No progressive decrease in the concentration of growth factors for L.leichmannii was found along the length of the small intestine in the pernicious anaemia patients, but the quantities involved were too small for any conclusion to be reached as to whether or not the defective secretion of patients with pernicious anaemia fails to protect vitamin B_{12} from "destruction" by bacteria in the gastrointestinal tract.

The source of both the factors for the growth of S.faecalis and L.leichmannii in the stomachs and small intestines of these patients is not obvious. It would seem that they might come from the intestinal secretions themselves, from the products of bacterial synthesis in the small intestine, or from the remains of previously ingested food matter. In view of the extremely small amounts of the growth factors found to be present, and the number of variables, further speculation on this subject would serve little purpose.

The dried weight of stool passed daily by the average adult is 25 to 50 G. Thus, from Table 25 it will be seen that, subject to the provision that some of the apparent folic acid may be citrovorum factor and that some of the apparent vitamin B_{12} may be factor A or pseudovitamin B_{12}, the stools of a case of pernicious anaemia may contain about 5 µg. of vitamin B_{12} and 0.5 mg. of folic acid daily. Davidson & Girdwood (1947) showed that 1 mg. of pteroylglutamic acid given orally daily was sufficient, in some patients with pernicious/

pernicious anaemia in relapse, to give a haemopoietic response, and numerous workers have obtained a response to 1 μg. of vitamin B_{12} given parenterally daily. Thus, it is obvious that the pernicious anaemia patient is producing large amounts of both haemopoietic factors in his large intestine, and does not differ in this from non-anaemic control patients. Moore (quoted by Spies, 1947) has shown that although patients can absorb enough folic acid when it is given by enema, the effective dose by this route is at least ten times that required by the oral route. As described on p.413 we had no response in one pernicious anaemia patient to the administration of 100 ml. of normal gastric juice together with 25 μg. of cyanocobalamin daily for four days by enema. There was a response to a similar dosage of these together by mouth.

These results for the "vitamin B_{12}" content of the stool are in fairly close agreement with those of Bethell et al. (1948), who selected pernicious anaemia patients on a normal diet. More recently Callender & Spray (1951) have obtained rather higher figures in the stools of pernicious anaemia patients (19, 3.8 and 22 μg. of "vitamin B_{12}" was the daily output).

The findings of this small piece of investigative work indicate that most of the "vitamin B_{12}" or "folic acid" in the faeces is produced by bacterial synthesis in the large intestine. Objections to this theory are at once obvious. We have already anticipated our later results by stating that the organisms isolated by us (and by others, working simultaneously along similar lines) from the stomach or small intestine of pernicious anaemia patients would under certain conditions

remove/

remove cyanocobalamin from the culture medium to which it had been added. It would therefore be reasonable to expect the faeces to contain very little vitamin B_{12}. There must however be remembered the reverse side of the picture, that Holbrook (1952) using L.leichmannii found that nearly all the organisms isolated from poultry litter produced "vitamin B_{12}". It will be recollected, too that cyanocobalamin is produced commercially from the culture broth of Streptomyces. It has also been prepared for commercial use by organisms isolated from the faeces of hens (Stokstad et al.,1948) and of horses (McSorley, 1951). To this must be added the findings of Ford (1952) who prepared factor C by growing B.coli in a vitamin B_{12}-free medium supplemented with factor B, and who found that factor A and vitamin B_{12} were recovered largely unchanged after passage through B.coli. Burkholder (1952) also was able to recover much of the cyanocobalamin that had been taken up by B.coli, and we have already seen that pseudovitamin B_{12} and pseudo-vitamin B_{12b} were prepared from anaerobic fermentation of an organism isolated from the bovine rumen. (Pfiffner et al., 1951, 1953). It may therefore be said that the faeces may contain vitamin B_{12} produced by micro-organisms, bacterial cells that have absorbed vitamin B_{12}, various other factors that may simulate vitamin B_{12} in certain assay techniques, and probably bacterial cells containing such substances. In addition interconversion of these substances by bacterial action probably occurs in the large intestine.

The next phase of this section of the investigation concerns the isolation of organisms from the stomach or small intestine and measurement of their effect in culture on haemopoietic factors.

SOME INVESTIGATIONS CONCERNING THE ABILITY OF MICROORGANISMS ISOLATED FROM THE ALIMENTARY TRACT TO PRODUCE OR REMOVE HAEMOPOIETIC FACTORS UNDER CULTURAL CONDITIONS.

PRELIMINARY INVESTIGATIONS

METHODS.

It should be noted that when these investigations were begun there was no published information on the subject to serve as a guide. At first only B.coli were investigated.

The preliminary investigations were done with the assistance of Dr. J. Somner, who passed sterile Ryle's tubes or weighted intestinal tubes in a series of patients, some of whom had pernicious anaemia. Dr. Somner isolated in sterile test tubes any organisms that were present, typed the B.coli, and supplied them to us for our investigations. After a number of experiments, it was decided to use nutrient broth as the basic medium for the growth of the organisms under investigation. This broth was made according to the method described by Mackie & McCartney (1948). It was found to be necessary to use more than one batch of the broth.

This culture medium was assayed on the occasion of each test for its own apparent content of the three haemopoietic factors, vitamin B_{12}, folic acid and citrovorum factor. A small measured quantity of each of these factors was added to separate flasks of the broth, the organisms under investigation were grown at 37° C. in the supplemented broth for 48 hours, the organisms in their culture fluid were centrifuged at high speed, and the supernatant, when clear, was assayed for its content of haemopoietic factor under consideration. Let us take/

take vitamin B_{12} as an example.

1. Organisms isolated from gastric juice, plated out on a MacConkey plate and any B.coli typed and grown on an agar slope. For example it may be found to be a Type I B.coli.

2. To the broth there is added 0.8 mμg. of cyanocobalamin per ml. The broth itself is found to contain 0.6 mμg. of vitamin B_{12} per ml. This is destroyable by alkaline hydrolysis and therefore should be vitamin B_{12}, but in case it might be some unknown substance other than true vitamin B_{12}, the cyanocobalamin has been added. This is of importance in relation to any possible disappearance of growth factors for L.leichmannii from the medium.

3. The Type I B.coli has been growing on an agar slope. A portion of a colony is diluted approximately a million fold, and 0.02 ml. of this diluted culture is added to the supplemented broth described at 2. above. It was found, in fact, that the size of the inoculum did not significantly affect the final result.

4. The B.coli is allowed to grow in the supplemented broth at 37° C. for about 48 hours.

5. This culture medium is now autoclaved at 15 lbs. pressure for 5 minutes, then centrifuged at 4,000 revs. for 20 minutes. If the supernatant is not clear, further centrifugation is carried out. The supernatant is then assayed for its content of vitamin B_{12} by means of the L.leichmannii technique. In the preliminary investigation, medium 2, p.171, was used.

The/

*The following information is relevant not only to the preliminary survey, but also to the later investigations, since such methods were used throughout.

For purposes of identification, the various strains of B.coli were tested with the fermentable substances glucose, lactose, dulcite, saccharose, manitol, maltose, and inositol, and their motility was noted. The other standard tests employed, further details of which may be found in textbooks of bacteriology were:-

Citrate utilisation test.

Atypical varieties of B.coli, unlike the typical ones, are able to use salts of organic acids as a source of carbon, and can grow in a synthetic medium containing sodium citrate. Growth in Koser's medium with visible turbidity within ten days indicates utilisation of the citrate. The medium is composed of:

1.5 G. Sodium ammonium hydrogen phosphate
1.0 G. Potassium dihydrogen phosphate
0.2 G. Magnesium sulphate
2.0 G. Sodium citrate.

This is made up in a litre of distilled water.

Gelatin liquefaction.

A stab culture is made and incubated at 22° C. for up to three weeks to see whether liquefaction occurs.

Production of indole.

It/

---

* The author wishes to express his thanks to Dr. A. F. McCabe of the Department of Bacteriology, University of Edinburgh, for advising about this part of the investigation.

It is necessary for tryptophane to be present in the medium, since it is from this that indole is produced. The organism is grown in peptone water at 37° C. for 24 hours. To the culture there is then added 1 ml. of ether, and the mixture is shaken. The ether is allowed to rise to the top of the tube to form a layer. To this there is added 0.25 ml. of Ehrlich's reagent A and 0.25 ml. of Ehrlich's reagent B.

Reagent A

Paradimethylamidobenzaldehyde	4 G.
96% alcohol	380 ml.
Concentrated hydrochloric acid	80 ml.

Reagent B

Potassium persulphate. Saturated watery solution.

If the test is positive, a rose red colour appears between the medium and ether, and spreads into the latter. If there is any doubt about the reaction, the rosindole compound may be extracted with amyl alcohol. If the reaction is not positive in 24 hours, a further tube is incubated for seven to ten days and then tested.

Voges and Proskauer's Reaction.

This reaction is given by atypical forms of B.coli. It is due to the formation of acetyl-methyl-carbinol from glucose. The organisms are grown for 48 hours at 30° C. in an 0.5% peptone water medium containing 0.5% glucose and 0.5% dipotassium hydrogen phosphate.

There is added 3 ml. of a 5% solution of α-naphthol in absolute alcohol and 1 ml. of 40% potassium hydroxide. If the reaction is positive, a pink colour appears in two to five minutes, a magenta or crimson colour in half an hour, and a deep crimson in one hour.

Methyl/

Methyl red Reaction.

After the organisms have been grown for 48 hours in glucose peptone water as described above, two drops of a methyl red solution are added. A red colour is considered to be positive, a yellow one negative.

Eijkman's test.

The organisms are grown in MacConkey's bile-salt lactose peptone water (with litmus indicator) at 37° C. and 44° C. A positive reaction occurs if acid and gas are produced.

The coliform strains may then be classified as follows.*

Type	Methyl Red	Voges & Prosk.	Citrate Util.	Indole Product	Eijkman 44° C.	Gelatin Liquef.
B.coli type I	+	-	-	+	+	-
B.coli type II	+	-	-	-	-	-
Intermed. type I	+	-	+	-	-	-
B.aerogenes type I	-	+	+	-	-	-
B.aerogenes type II	-	+	+	+	-	-
B.cloacae	-	+	+	-	-	+
Irregular I, coli-like I	+	-	-	+	-	-
Irregular II, coli-like II	+	-	-	-	+	-
Irregular III, coli-like III	+	-	-	-	-	+
Irregular IV, intermed.-like	+	-	+	-	-	+
Irregular V, aerogenes-like I	-	+	-	-	-	-
Irregular VI, aerogenes-like II	-	+	+	-	+	-
Irregular VII	-	-	+	+	-	-
Irregular VIII	-	-	-	-	-	-
Intermed. type II	+	-	+	+	-	-

B.coli mutabilis produces acid and gas with glucose but the other reactions are negative except that there may be slow production of acid and gas with lactose.

---

* From information supplied by Dr. A. F. MacCabe.

In the preliminary investigation, the following types of B.coli were isolated and tested.

Case 1. Pernicious anaemia in relapse. Tube reached jejunum. Irregular I, Irregular II, Type I and Type II B.coli isolated.

Case 2. Pernicious anaemia in relapse. Tube reached jejunum. Type I and Irregular II B.coli isolated. Twenty colonies of these two types were taken from a MacConkey plate and separately examined for their ability to produce haemopoietic factors in the culture medium or to remove them from it.

Case 3. Pernicious anaemia in relapse. Gastric contents. Intermediate I B.coli isolated.

Case 4. Pernicious anaemia in relapse. Gastric contents. Irregular V and Aerogenes II isolated.

Case 5. Pernicious anaemia in relapse. Intermediate B.coli isolated.

Case 6. Pernicious anaemia in relapse. Type II B.coli isolated.

No B.coli were isolated from the gastric juice of the following patients:

One with gastric carcinoma and hypochlorhydria,
One with untreated pernicious anaemia,
One with pernicious anaemia, a week after the commencement of cyanocobalamin therapy,
Two with pernicious anaemia, on long continued treatment with cyanocobalamin,
Two with megaloblastic anaemia of pregnancy,
One with haemolytic anaemia,
Three with no anaemia and with a normal amount of hydrochloric acid in the gastric juice.

The results obtained in this preliminary investigation are shown in Tables 26a and b.

TABLE 26a.

Preliminary Investigation of Effect on Quantities of Haemopoietic Factors of Growing Strains of B.coli in Nutrient Broth.

Case	Type of B.coli	Factor	Quantity of Haemopoietic Factors (mμg./ml.) Broth content	Supplement	Broth + Supplement	Quantity after Organisms grown
1	Irreg. I	B_{12}	0.6	0.8	1.2	3.4
		FA	12.0	16.7	36.0	41.0
		CF	12.5	16.7	25.8	31.8
	Irreg. II	B_{12}	0.6	0.8	1.2	3.5
Jejunal		FA	12.1	16.7	34.2	64.7
contents		CF	12.8	16.7	27.0	31.0
	Type I	B_{12}	0.6	0.8	1.2	3.9
		FA	12.1	16.7	34.2	40.0
		CF	12.8	16.7	27.0	30.0
	Type II	B_{12}	0.6	0.8	1.2	3.3
		FA	12.1	16.7	34.2	39.0
		CF	12.8	16.7	25.8	30.2
2	Type I	B_{12}	0.5	0.8	1.1	5.5
Jejunal		FA	14.7	16.7	30.0	26.2
contents		CF	10.0	16.7	23.0	28.0
	Irreg.II	B_{12}	0.5	0.8	1.1	3.9
		FA	14.7	16.7	30.0	31.0
		CF	10.0	16.7	23.0	22.0
3	Intermed.	B_{12}	2.3	0.8	3.1	5.8
Gastric	I	FA	5.6	16.7	21.0	45.4
contents		CF	27.0	16.7	43.0	49.0
4	Irreg. V	B_{12}	2.3	0.8	3.1	6.0
Gastric		FA	5.6	16.7	21.0	27.4
contents		CF	27.0	16.7	43.0	49.0
	Aerogenes	B_{12}	2.3	0.8	3.1	5.7
	II	FA	5.6	16.7	21.0	24.4
		CF	27.0	16.7	43.0	58.0
5	Intermed.	B_{12}	2.3	0.8	3.1	6.2
Gastric	I	FA	5.6	16.7	21.0	25.4
contents		CF	27.0	16.7	43.0	49.0
6	Type II	B_{12}	2.3	0.8	3.1	8.4
Gastric		FA	5.6	16.7	21.0	22.6
contents		CF	27.0	16.7	43.0	52.0

In Table 26a, for the sake of simplicity, there have been included in relation to Case 2 the findings for only one colony of Type I. B.coli and one of Irregular II. In fact, however, a total of twenty colonies were investigated. The results were as shown in Table 26b.

TABLE 26b.

Quantity of Haemopoietic Factors present after Culturing Organisms from Twenty separate Colonies of B.coli in Nutrient Broth.

Case 2. (mμg./ml. in all instances)

	Broth Content	Added Supplement	Broth + Supplement
B_{12}	0.5	0.8	1.1
	14.7	16.7	30.0
	10.0	16.7	23.0

Quantity present after growth of organisms

Type I. B.coli (9 colonies)

B_{12} 5.5, 3.4, 4.7, 3.6, 3.5, 8.9, 5.0, 3.5, 5.3

FA 26.2, 31.0, 32.4, 34.0, 32.2, 36.0, 33.2, 32.0, 34.0,

CF 28.0, 24.0, 22.6, 23.0, 23.8, 23.0, 24.0, 22.0, 23.3

Irreg. II B.coli (11 colonies)

B_{12} 3.9, 5.6, 8.3, 3.7, 10.0, 8.6, 4.6, 4.7, 3.4, 3.4, 3.8

FA 31.0, 32.0, 34.0, 30,0, 33.4, 38.0, 36.0, 32.0, 31.0, 26.2, 32.6

CF 22.0, 24.6, 22.5, 23.8, 24.0, 25.0, 22.6, 25.0, 24.6, 20.6, 22.0

The data shown in Tables 26a and 26b suggest that all these strains of B.coli were able, under the cultural conditions present, to produce a substance that was a growth factor for L.leichmannii, although the amount produced differed with different colonies of the same type.

It/

It was shown further that this substance was destroyed by alkaline hydrolysis, and the next step was to see whether it was a growth factor for the mutant of B.coli used in our other assay method for vitamin B_{12} (p.172). The results of this investigation were as shown in Table 27.

TABLE 27.

Comparison of apparent Vitamin B_{12} produced by Strains of B.coli in culture, two Assay Methods being used.

Case	Assay Method used	Type of B.coli tested for production of haemopoietic factors	Quantity of Haemopoietic Factors (mμg./ml.) Broth + added Supplement	Quantity after Organisms grown
3	L.leichmannii	Intermed. I.	3.1	5.8
	B.coli		2.7	3.8
4	L.leichmannii	Irreg. V	3.1	6.0
	B.coli		2.8	3.5
4	L.leichmannii	Aerogenes II	3.1	5.7
	B.coli		2.8	2.8
5	L.leichmannii	Intermed. I	3.1	6.2
	B.coli		2.8	3.3
6	L.leichmannii	Type II	3.1	8.4
	B.coli		2.8	3.3

The results shown in Table 27 suggest that much of the apparent vitamin B_{12} produced is not vitamin B_{12}. It may be pseudovitamin B_{12} (see p.202).

It will be seen from Tables 26a and 26b that there was little evidence of production of folic acid or citrovorum factor except that the Irregular II strain in Case 1 and the Intermediate I strain in Case 3 appeared to produce folic acid. This substance was folic acid itself or an allied substance rather/

rather than citrovorum factor, because further experiments showed that the substance would not support the growth of L.citrovorum.

The strain Irregular II of Case 1 was subcultured for a period of two months and was still capable of producing folic acid. It was then subcultured for a further two months and no longer appeared able to do this.

PRELIMINARY INVESTIGATIONS USING MIXED ORGANISMS.

Unfortunately Dr. J. Somner had to cease her work on the isolation of organisms early in 1953, and it was then decided in the first instance to investigate the behaviour of mixed organisms from the alimentary tract in dealing with haemopoietic factors while being cultured. In these experiments 0.1 ml. of fasting gastric juice or of duodenal contents was dropped into several flasks containing nutrient broth, which in each instance was supplemented with one of the three haemopoietic factors. One of each of these was incubated in the usual way for 48 hours and a second flask was incubated under anaerobic conditions for 72 hours. The results obtained are shown in Table 28.

TABLE/

TABLE 28.

Haemopoietic Activity of Mixed Organisms from Gastric or Duodenal Contents.

			Apparent B_{12} mμg./ml.	Folic Acid Activity mμg./ml.	Citrovorum Factor mμg./ml.
Broth content			0.3	8.0	17.3
Added supplement			0.8	16.7	16.7
Broth + supplement			1.2	29.0	30.0
Content following growth of mixed flora					
Case		Method of Culture			
7	PA Gastric contents	aerobic	3.6	20.6	29.6
		anaerobic	3.0	21.0	29.0
8	Meg. An. of Preg. Gastric contents	aerobic	2.4	31.0	29.0
		anaerobic	2.9	14.8	27.6
9	PA Gastric contents	aerobic	12.0	42.2	32.0
		anaerobic	20.0	46.0	31.1
10	PA Gastric contents	aerobic	2.6	-	23.4
		anaerobic	2.2		24.1
11	Neurosis Duodenal contents	aerobic	5.3	22.0	52.8
		anaerobic	5.0	29.0	38.0

None of the mixed flora shown in Table 28 were able under the conditions of the experiment to remove haemopoietic factors from the medium with the possible exception of Case 8 in relation to folic acid under anaerobic conditions. There was evidence that all the mixed flora could produce a growth factor/

factor for L.leichmannii, that the organisms in Case 9 could possibly produce one for S.faecalis, and that those in Case 11 could produce one for L.citrovorum. None of these gastric juices contained B.coli in that there was no growth when the contents of the stomach or duodenum were plated on MacConkey plates in various dilutions. This was done without delay when the samples were obtained. The flora was not investigated further in these cases.

The conclusion from these preliminary and purposely superficial investigations was that under the conditions of the investigation, all strains of B.coli isolated from the upper alimentary tract and transferred from agar slopes to the cultural medium produced a growth factor or factors for L.leichmannii. Most of this was not vitamin B_{12}-but possibly pseudovitamin B_{12}. Mixed flora other than B.coli, if introduced directly into the cultural medium had similar properties. The effects in relation to folic acid or citrovorum factor were inconstant.

Further Investigations.

So far the investigations had been of a superficial preliminary nature, but subsequent work was much more complex.

In the preliminary work organisms had been taken from agar slopes or directly from gastric contents and it was likely that different results would be obtained if the organisms were subcultured, particularly if this was done in a medium deficient in haemopoietic factors. There was the further complication that the conditions of the investigation were not now identical in that fresh supplies of nutrient broth/

broth had to be obtained, and that the hydrolysed casein of the L.leichmannii assay had to be replaced by another batch, and other constituents of the medium to be replenished. Since serum assays for vitamin B_{12} were now being done simultaneously it was convenient to use medium 3 (p.171) both for the estimations of serum vitamin B_{12} level and for the investigations of bacterial action. Duplicate experiments showed that similar results were obtained with the subcultured organisms whether medium 2 or medium 3 was used.

About this time a paper by Burkholder (1952) came to our notice. This worker too had been isolating strains of bacteria from the stomach and jejunal contents of pernicious anaemia patients and had first grown them in a vitamin B_{12} deficient medium, then transferred them to the same medium supplemented with cyanocobalamin. He claimed that most of the bacteria had a strong tendency to absorb vitamin B_{12} from solution. Burkholder spun down the culture of organisms in his medium with its added vitamin B_{12}, and then autoclaved the supernatant rather than autoclaving the culture before centrifugation.

Accordingly our investigations were extended to include studies involving the use of Burkholder's medium. Its composition is as follows:-

$K_2 HPO_4$	7.0 G.
$K H_2 PO_4$	3.0 G.
Na citrate $3H_2 O$	0.5 G.
$Mg SO_4 \ 7H_2O$	0.1 G.
$(N H_4)_2 SO_4$	1.0 G.
Dextrose	10.0 G.
Asparagine	4.0 G.
Water	to 1,000 ml.
Adjust pH to 6.8	

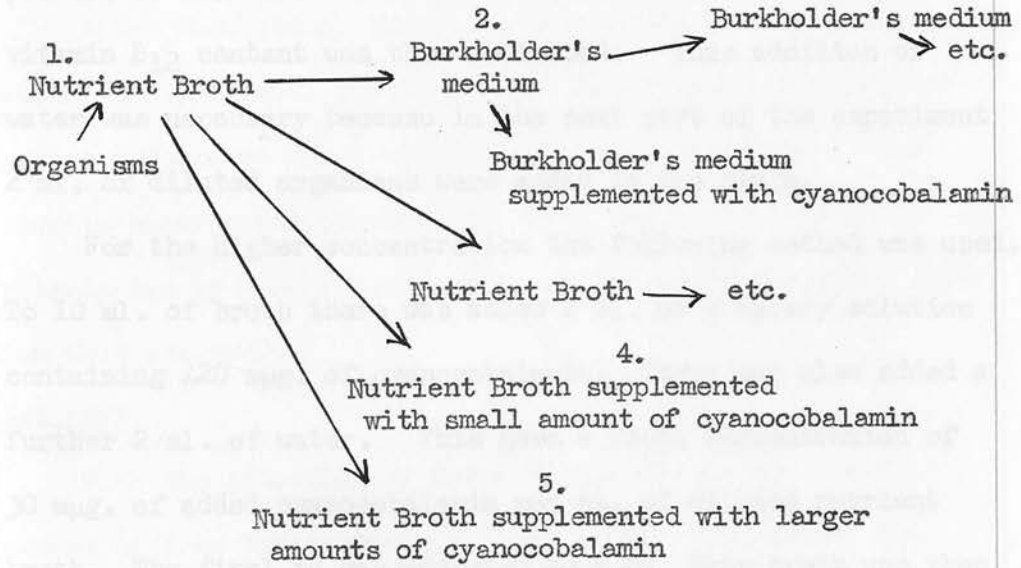
INVESTIGATION/

INVESTIGATION No. 1.

Here the project was to inoculate nutrient broth with mixed organisms from the gastric juice and grow them for 24 hours.

From this they were subcultured for 48 hours in nutrient broth supplemented with two levels of cyanocobalamin and also for 24 hours in Burkholder's medium. From this Burkholder's medium they were subcultured for 48 hours in Burkholder's medium supplemented with cyanocobalamin. In each instance the broth was assayed for its content of vitamin B_{12} before and after the organisms were grown in it. The organisms were identified and thereafter repeatedly subcultured in nutrient broth,,on agar slopes and in Burkholder's medium for later investigations. It was found that many species of organisms soon died out in Burkholder's medium.

The method employed may be summarised as follows:-



The arrows indicate transfer of organisms.

This/

This involved in each instance ten assays of media for their vitamin B_{12} content, since the media numbered 1. to 5. above had to have this estimated each time before and after the organisms had been cultured in them. The effect of repeated subculturing on the mixed flora is considered later.

The details of the method were as follows:-

IDENTIFICATION OF ORGANISMS.

These were plated out on blood agar plates and on MacConkey's medium. They were stained with Gram's stain and examined microscopically. Identification of types of B.coli was by the methods already described.

QUANTITY OF CYANOCOBALAMIN ADDED.

Nutrient Broth. Two levels of cyanocobalamin were added. Where the smaller quantity was used, the method was to take 12 ml. of broth containing 0.83 mμg. of added cyanocobalamin per ml. of mixture. To this 2 ml. of water was added and the vitamin B_{12} content was then estimated. This addition of water was necessary because in the next part of the experiment 2 ml. of diluted organisms were added in its place.

For the higher concentration the following method was used. To 10 ml. of broth there was added 2 ml. of a watery solution containing 420 mμg. of cyanocobalamin. There was also added a further 2 ml. of water. This gave a final concentration of 30 mμg. of added cyanocobalamin per ml. of diluted nutrient broth. The final pH was adjusted to 6.8. This broth was then assayed for its vitamin B_{12} content. Again the 2 ml. of water had been added because in the next part of the investigation it

was/

was replaced by 2 ml. of diluted organisms.

Burkholder's medium. This was treated as was the nutrient broth in the second instance above, there being a final concentration of 30 mμg. of cyanocobalamin per ml. of incubating mixture. The pH was adjusted to 6.8. The medium was assayed for its vitamin B_{12} content.

ADDITION OF ORGANISMS.

The inoculum was prepared by centrifuging a 24 hour broth culture and resuspending the cells in the basal medium to be employed for absorption experiments, so as to yield approximately a tenfold concentration. Two ml. of inoculum were added in place of the 2 ml. of water to the nutrient broth with and without added cyanocobalamin (both levels) and to the Burkholder's medium with and without added cyanocobalamin.

Incubation with these concentrated bacterial suspensions was allowed to proceed for 24 hours at 37° C.

After incubation, the bacteria were removed by centrifugation at 4,000 revs. per minute for 20 minutes, and the supernatant autoclaved for 5 minutes at 15 lbs. pressure. The sterile supernatant liquors were then tested for residual vitamin B_{12} by microbiological assay with L.leichmannii; medium No.3 was used.

PATIENTS.

With the exception of Cases 21 and 22 who had a small amount of free hydrochloric acid in the gastric juice, the following patients had histamine fast achlorhydria. Where serum vitamin B_{12} levels are indicated, this was by the L.leichmannii/

L.leichmannii method described in Chapter 8. The normal level is 140 - 750 μμg./ml.

Case 12.

Gastric juice of a patient with untreated pernicious anaemia and no evidence of subacute combined degeneration of the cord. Hb. 7.7 G. per 100 ml., red cells 1,900,000 per c.mm. Serum vitamin B_{12} level 120 μμg./ml.

Case 13.

Gastric juice of a patient with untreated pernicious anaemia and no evidence of subacute combined degeneration of the cord. Hb. 6.7 G. per 100 ml., red cells 2,000,000 per c.mm. Serum vitamin B_{12} level 95 μμg./ml.

Case 14.

Gastric juice from a patient with treated pernicious anaemia complicated by advanced gastric carcinoma. Hb. 10.4 G. per 100 ml., red cells 4,050,000 per c.mm.

Case 15.

Gastric juice from a patient with regional jejunitis and untreated megaloblastic anaemia (Case 15, p.364). Hb. 10.1 G. per 100 ml., red cells 3,710,000 per c.mm. Serum vitamin B_{12} level 175 μμg./ml.

Case 16.

Gastric juice from a patient with untreated idiopathic steatorrhoea and megaloblastic anaemia (Case 18, p.370). Hb. 7.0 G. per 100 ml., red cells 3,070,000 per c.mm. Serum vitamin B_{12} level 160 μμg./ml.

Case 17.

Gastric juice from a case of idiopathic steatorrhoea with megaloblastic/

megaloblastic anaemia despite liver injections. Hb. 5.6 G. per 100 ml., red cells 3,280,000 per c.mm. (Case 17, p.368)

Case 18.

Gastric juice from a patient with idiopathic steatorrhoea and untreated megaloblastic anaemia. Hb. 8.9 G. per 100 ml., red cells 2,800,000 per c.mm.

Case 19.

Gastric juice from a patient with Raynaud's phenomenon and what appeared to be severe peripheral neuritis. Hb. 9.9 G. per 100 ml., and red cells 4,010,000 per c.mm. The marrow was normoblastic and the serum vitamin B_{12} level was 330 μμg./ml.

Case 20.

Gastric juice from a patient with clinical features suggesting pernicious anaemia, a serum vitamin B_{12} level of 50 μμg./ml., a haemoglobin level of 10.1 G. per 100 ml. and a red cell count of 2,700,000 per c.mm., but a completely normoblastic marrow without treatment. (Graph 33, p.428).

Case 21.

Gastric juice from a patient with untreated megaloblastic anaemia of pregnancy. Hb. 8.2 G. per 100 ml., red cells 2,100,000 per c.mm. The serum vitamin B_{12} level was 170 μμg./ml.

Case 22.

Gastric juice from a patient with untreated megaloblastic anaemia of pregnancy. Hb. 9.9 G. per 100 ml., red cells 2,520,000 per c.mm. The serum vitamin B_{12} level was 260 μμg./ml.

Case 23./

Case 23.

Small intestinal contents obtained at operation from a stagnant loop in a patient with treated megaloblastic anaemia associated with an ileo sigmoid fistula. The patient had recently been treated with cyanocobalamin injections but prior to operation had received streptomycin and sulphasuccidine (case 208, p.545).

The results of these various investigations are given in Table 29.

It will be seen from Table 29 that in all instances vitamin B_{12} disappeared from the nutrient broth to a greater or less extent, except in Cases 17 (Staphylococci), possibly 19 (Staphylococci and streptococci) and in Case 23. The organisms in the small intestinal contents of Case 23 were B.coli, intermediate I, but it will be recollected that pre-operative treatment with streptomycin and sulphasuccidine had been given.

Removal of vitamin B_{12} from the medium was less complete in Burkholder's medium than it was in nutrient broth.

It was found further that if the organisms known to cause vitamin B_{12} to disappear from the medium were allowed to grow in it as before, and were killed by autoclaving and the medium with dead organisms was then assayed for its vitamin B_{12} content without centrifugation and separation of the supernatant, vitamin B_{12} was not shown to have disappeared. Since the assay itself involves a 1 in 200 dilution of the previously incubated medium, this was vitamin B_{12} or a related substance that was being measured and not turbidity produced by the dead organisms. This indicated that the organisms did not destroy vitamin B_{12}, but merely absorbed or adsorbed it.

TABLE/

TABLE 29.

Results of Incubating Organisms with Media with and without added Cyanocobalamin.

Content of growth factors for L.leichmannii mμg./ml.

Case No. & Organisms present & added to incubating mixture	Nut. Broth (N.B.)	N.B. + 0.7 mμg. B_{12}/ml.	N.B. + Org. *	N.B. + 0.7 mμg. B_{12} + Org.	N.B. + 30 mμg. B_{12}/ml.	N.B. + 30 mμg. B_{12} + Org.	BM **	BM + 30 mμg. B_{12}/ml.	BM + 30 mμg. B_{12} + Org.	BM + Org.
12 B.coli Intermed.II	1.0	1.9	0.5	0.6	30.0	8.0	Nil	32.4	24.0	Nil
13 B.aerogenes I Staph.	2.5	3.3	1.7	0.4	31.5	1.6	Nil	29.0	30.0	Nil
14 B.coli Intermed.II yeasts	1.0	1.7	0.3	0.6	30.1	3.6	Nil	30.0	13.7	Nil
15 B.aerogenes I Staph. Strep.	2.8	3.6	0.2	0.2	35.4	0.4	Nil	30.4	15.4	Nil
16 Staph. B.coli Mutabilis	2.4	3.1	1.1	1.0	28.8	3.0	Nil	29.0	22.0	Nil
17 Staph.	2.8	3.7	2.3	3.3	32.0	32.0	Nil	28.4	28.4	Nil
18 B.coli Type I Staph.	2.9	4.1	0.1	0.1	31.6	Negl.	Nil	28.4	13.8	Nil
19 Staph. Strep.	3.0	3.6	2.0	2.2	31.4	25.4	Nil	29.0	28.4	Nil
20 B.coli Type I	1.0	1.9	0.4	0.8	36.0	11.6	Nil	33.0	15.1	Nil
21 B.cloacae Staph. Strep.	4.2	4.6	0.2	0.2	32.4	1.0	Nil	32.0	3.2	Nil
22/										

* Org. = Added organisms
** BM = Burkholder's medium

Case No. & Organisms present & added to incubating mixture	Nut. Broth (N.B.)	N.B. + 0.7 mμg. B_{12}/ ml.	N.B. + Org. *	N.B. + 0.7 mμg. B_{12} + Org.	N.B. + 30 mμg. B_{12}/ ml.	N.B. + 30 mμg. B_{12} + Org.	BM **	BM + 30 mμg. B_{12}/ ml.	BM + 30 mμg. B_{12} + Org.	BM + Org.
22 B.coli Intermed.II Staph. Diphtheroids	3.8	4.3	0.2	0.6	28.4	1.4	Nil	32.0	2.4	Nil
23 B.coli Intermed.I	2.1	2.7	1.7	2.2	32.0	32.0	Nil	29.0	29.4	Nil

*Org. = Added organisms

**BM = Burkholder's medium

INVESTIGATION No.2.

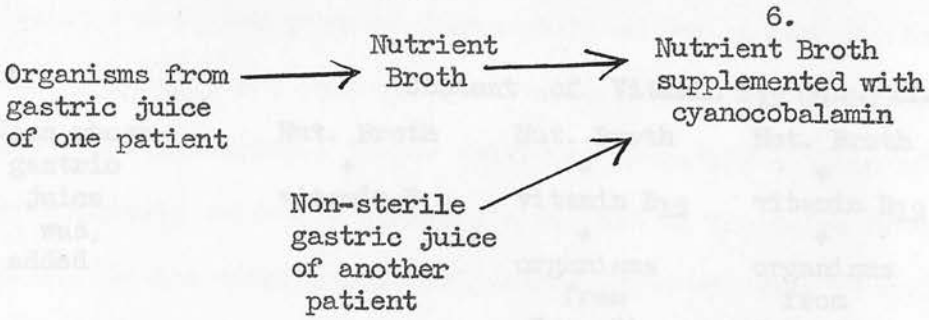
Since one view about the mode of action of intrinsic factor is that it interferes with the uptake of vitamin B_{12} by bacteria in the alimentary tract, the next phase of this investigation was to see whether or not gastric juice was capable of doing this in culture medium. Burkholder (1952) using his medium referred to above found that normal gastric juice inhibited the uptake of vitamin B_{12} by intestinal bacteria and stated:

> "It appears that appreciable amounts of the vitamin are absorbed by bacteria growing under experimental conditions not unlike those in the gastrointestinal region of the pernicious anaemic, but in the presence of normal gastric juice this absorption is inhibited."

In fact, however, it is stretching the imagination considerably to suggest that a single strain of organism growing in a medium consisting of a mixture of phosphates, sodium citrate, magnesium and ammonium sulphate, dextrose and asparagine in a flask reproduces conditions in the gastrointestinal region. We preferred in the first instance to investigate the problem by using nutrient broth as culture medium, but did not consider that the conditions in the alimentary tract were being closely reproduced. The object was to subculture organisms from nutrient broth to nutrient broth supplemented with cyanocobalamin and to see whether normal or pernicious anaemia gastric juice had any effect on the uptake of vitamin B_{12} by the organisms. In the first instance no attempt was made to sterilise the gastric juice because it was thought that organisms introduced with/

with the gastric juice should themselves be prevented from taking up vitamin B_{12} by intrinsic factor or whatever part of the gastric juice performed this function.

The plan was as follows:



This involved assaying 6. for its vitamin B_{12} content under three conditions

Nutrient broth + cyanocobalamin
Nutrient broth + cyanocobalamin + organisms
Nutrient broth + cyanocobalamin + normal gastric juice + organisms.

The details of the method were as follows:

The three mixtures consisted, respectively, of

10 ml. nutrient broth + 2 ml. of a watery solution containing 500 mμg. of cyanocobalamin + 2 ml. of water. This contained approximately 36 mμg. of vitamin B_{12} per ml.

8 ml. nutrient broth + 2 ml. of a watery solution containing 500 mμg. of cyanocobalamin + 2 ml. of water + 2 ml. of organisms prepared as on p.229 and suspended in nutrient broth.

8 ml. nutrient broth + 2 ml. of a watery solution containing 500 mμg. of cyanocobalamin + 2 ml. of gastric juice being tested + 2 ml. of organisms prepared as above.

These were each incubated for 24 hours at 37° C. after the pH had been adjusted to 6.8 After incubation and centrifugation as before, the supernatant was autoclaved and tested for residual vitamin B_{12} by microbiological assay with L.leichmannii; medium No.3 was used.

The results are given in Table 30.

TABLE 30.

Effect of non-sterile Gastric Juice on Uptake of of Vitamin B_{12} by B.coli Type I, isolated for from the Gastric Juice of Case 20 (p. 231)

Case whose gastric juice was added	Content of Vitamin B_{12} (mμg./ml.) Nut. Broth + vitamin B_{12}	Nut. Broth + vitamin B_{12} + organisms from Case 20	Nut. Broth + vitamin B_{12} + organisms from Case 20 + gastric juice
12 (PA)	35.4	13.5	27.0
13 (PA)	27.5	11.3	13.2
14 (PA and gastric carcinoma)	36.0	10.8	31.1
16 (Id.Steatorrhoea)	27.5	7.0	7.8
17 (Id.Steatorrhoea)	28.4	13.5	28.5
18 (Id.Steatorrhoea)	28.6	13.3	29.2
19 (Periph.Neuritis)	28.6	11.3	21.6
21 (Meg.An.of Preg.)	35.4	13.0	32.4
22 (Meg.An.of Preg.)	36.0	4.1	8.9

It will be seen that the results in Table 30 do not indicate that under the circumstances of our assay and with the strain of B.coli used, a test for intrinsic factor has been devised. It is true that it might be argued that in Cases 17, 18, 19 and 21 the gastric juice inhibited the uptake of vitamin B_{12} by B.coli, and that the gastric juice of Cases 16 and 22 did not contain intrinsic factor (although there was a trace of free hydrochloric acid with Gunzberg's reagent in the juice of Case 22). On the other hand, the gastric juice of Cases 12 and 14 seemed to prevent the uptake of vitamin B_{12} by bacteria, and there was no reason to believe that any intrinsic factor was present there.

If the matter is considered from the bacteriological point of view, remembering that non-sterile gastric juice was used, the juices that appeared to give some protection were those of:-

Case	Organisms present
12	B.coli
14	B.coli, yeasts
17	Staphylococci
18	B.coli, staphylococci
19	Staphylococci, streptococci
21	B.cloacae, staphylococci, streptococci

Those that did not protect were:-

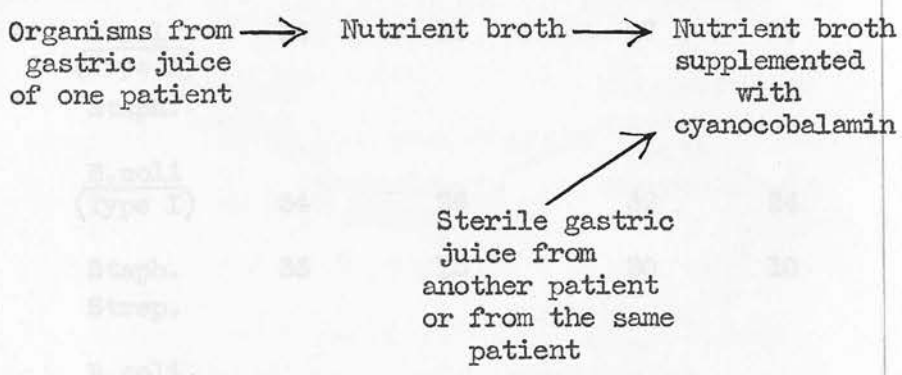
13	B.coli, staphylococci
16	B.coli, staphylococci
22	B.coli, staphylococci, diphtheroids

This did not appear to give any information of value. It could not be shown from these results that addition of two strains of organisms that could remove vitamin B_{12} from the culture medium necessarily gave a combination against which gastric juice was powerless.

In short, this test was of no practical value.

INVESTIGATION No.3.

The next stage was to sterilise gastric juice and then see what was its capacity to prevent the uptake of vitamin B_{12} by organisms isolated from the alimentary tract. This could only be done by Berkefeld filtration, since it is generally agreed that any other method will destroy intrinsic factor or remove it from the gastric juice. The techniques were as in Investigation No.2, except that the gastric juice had been filtered. The plan was as follows:-



For this investigation, the gastric juices used were from Cases 14 (P.A. and Gastric Carcinoma) and 22 (Megaloblastic anaemia of pregnancy). The organisms used were from Cases 18 (Idiopathic steatorrhoea), 20 (Macrocytic anaemia with low serum vitamin B_{12} level), 21 (Megaloblastic anaemia of pregnancy) and 22 (Megaloblastic anaemia of pregnancy). The variety of organisms now present after repeated subculturing were checked, and it was found that B.cloacae had disappeared from the subculture of Case 21.

The results are given in Table 31.

These results were not very helpful. It seemed that in some instances the organisms were losing their capacity to absorb/

TABLE 31.

Effect of Sterile Gastric Juice on Uptake of Vitamin B_{12} by Organisms Isolated from Gastric Juices.

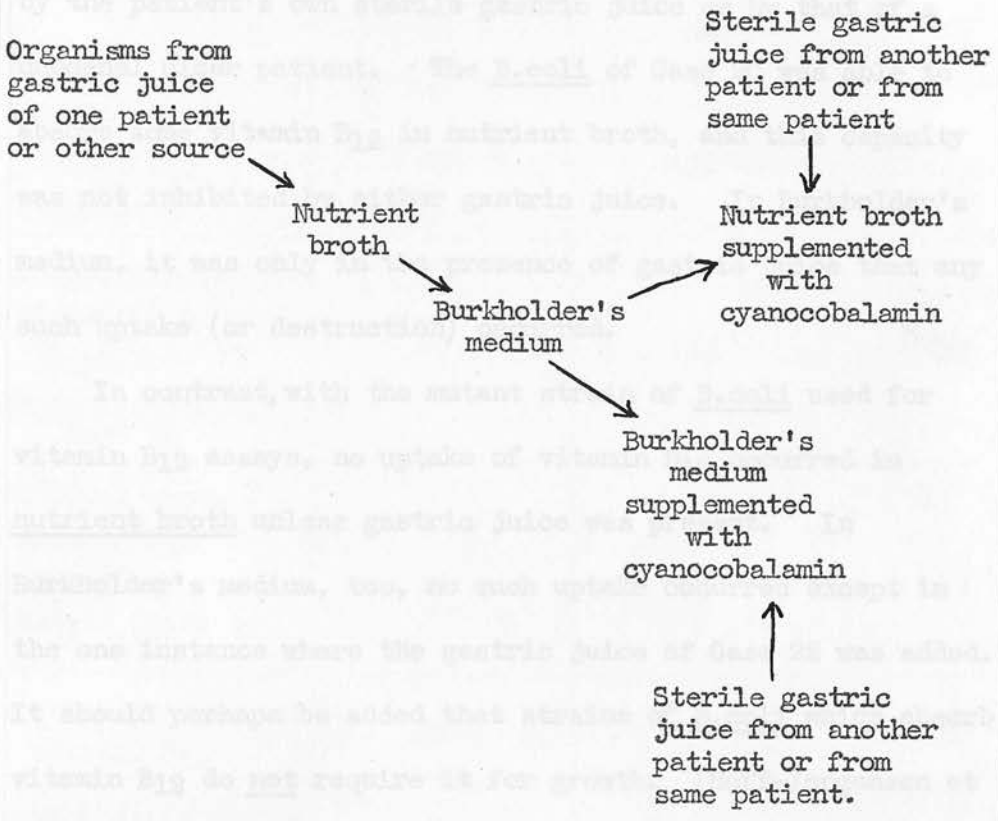
Case whose organisms were used	Organisms present	Content of vitamin B_{12} (mμg./ml.) Nutrient broth + Vit.B_{12}	Nutrient broth + Vit.B_{12} + Organisms	Nutrient broth + Vit.B_{12} + Organisms + gastric juice of Case 14	Nutrient broth + Vit.B_{12} + Organisms + gastric juice of Case 22
18	B.coli (Type I) Staph.	34	16	17	20
20	B.coli (Type I)	34	26	32	24
21	Staph. Strep.	33	13	20	10
22	B.coli (Intermed.II) Staph. Diphtheroids	35	36	33	43

vitamin B_{12}, and that, if anything, the gastric juice of the pernicious anaemia case (Case 14) was more able to prevent any such uptake than that of the megaloblastic anaemia of pregnancy case. (The other gastric juices were less suitable for Berkefeld filtration.)

INVESTIGATION No.4.

The obvious investigation to carry out now was to attempt to make the organisms vitamin B_{12} deficient by subculturing them in Burkholder's medium, and to obtain a more definitely normal gastric juice to measure its possible inhibitory effect on the uptake of vitamin B_{12} by the organisms.

The plan was as follows:-



For this investigation the gastric juices used were from Case 22 (megaloblastic anaemia of pregnancy) and from a new patient, Case 23, who suffered from a duodenal ulcer.

The organisms were:

(1) From Case 20 (macrocytic anaemia with low serum vitamin B_{12} level) *B.coli*, Type I.

(2) From Case 22 (megaloblastic anaemia of pregnancy) *B.coli*, Intermediate II, Staphylococci, Diphtheroids.

(3)/

(3) the mutant strain of B.coli used for the vitamin B_{12} assay as described on p.172.

The techniques were as already described. The results are given in Table 32.

The results shown in Table 32 suggest that the mixed organisms from Case 22, by virtue of their passage through Burkholder's medium, had had restored to them their capacity to absorb vitamin B_{12} but that this capacity was not overcome by the patient's own sterile gastric juice or by that of a duodenal ulcer patient. The B.coli of Case 20 was able to absorb some vitamin B_{12} in nutrient broth, and this capacity was not inhibited by either gastric juice. In Burkholder's medium, it was only in the presence of gastric juice that any such uptake (or destruction) occurred.

In contrast, with the mutant strain of B.coli used for vitamin B_{12} assays, no uptake of vitamin B_{12} occurred in nutrient broth unless gastric juice was present. In Burkholder's medium, too, no such uptake occurred except in the one instance where the gastric juice of Case 22 was added. It should perhaps be added that strains of B.coli which absorb vitamin B_{12} do not require it for growth. (Hoff-Jorgensen et al, 1952). The same results with the mutant strain of B.coli were obtained in a duplicate experiment, and the only possible conclusions would seem to be that under the conditions of these investigations (which are quite different from the conditions of the assay for vitamin B_{12} with this organism), the organism can either grow without absorbing sufficient vitamin B_{12} to give a significant change in the result obtained (by using only 1 or 2 mμg. per ml.), or, less likely, that they do not require vitamin/

TABLE 32.

Effect of Sterile Gastric Juice on Uptake of Vitamin B_{12} by Organisms that had been subcultured in Burkholder's medium.

Source of Organisms →	Case 20 (B.coli, type I)	Case 22 B.coli, intermed. II Staph. Diphtheroids	Mutant strain of B.coli
	Content of vitamin B_{12} (mμg./ml.)		
Nutrient broth + vitamin B_{12}	34	30	35
Nutrient broth + vitamin B_{12} + organisms	25	11	34
Nutrient broth + vitamin B_{12} + organisms + gastric juice of Case 22	20	14	27
Nutrient broth + vitamin B_{12} + organisms + gastric juice of Case 23	23	10	18
Burkholder's medium + vitamin B_{12}	36	37	36
Burkholder's medium + vitamin B_{12} + organisms	36	15	34
Burkholder's medium + vitamin B_{12} + organisms + gastric juice of Case 22	20	26	22
Burkholder's medium + vitamin B_{12} + organisms + gastric juice of Case 23	16	15	34

vitamin B_{12} for such growth.

The matter did not appear to merit further investigation. The main features appeared to be that a test for intrinsic factor had not been obtained, and that the capacity of organisms to absorb vitamin B_{12} (e.g. those of Case 22) may vary according to their previous environmental conditions.

INVESTIGATION No.5.

It was thought that it would be of interest in this respect to see whether some of the other organisms had altered their capacity to absorb the vitamin. These organisms had been repeatedly subcultured in nutrient broth, and for the purposes of this small investigation they were not passed through Burkholder's medium. As a variant, and because of trouble that had developed with the growth of the standard in Medium No.3, Medium No.2 was now used. On this occasion the smaller amount of vitamin B_{12} (0.8 mμg./ml.) was added.

The results obtained are given below in mμg./ml. The first reading is for the vitamin B_{12} content of the nutrient broth supplemented with vitamin B_{12}, and the second is that following growth of the organisms. There is also given, from Table 29, the results previously obtained.

Case 14 B.coli, intermed.II, yeasts	Broth content	4.8	After growth	1.1	Formerly	1.7 : 0.6
Case 17 Staphylococci	Broth content	4.3	After growth	4.3	Formerly	3.7 : 3.3
Case 20 B.coli Type I	Broth content	4.3	After growth	0.3	Formerly	4.6 : 0.2

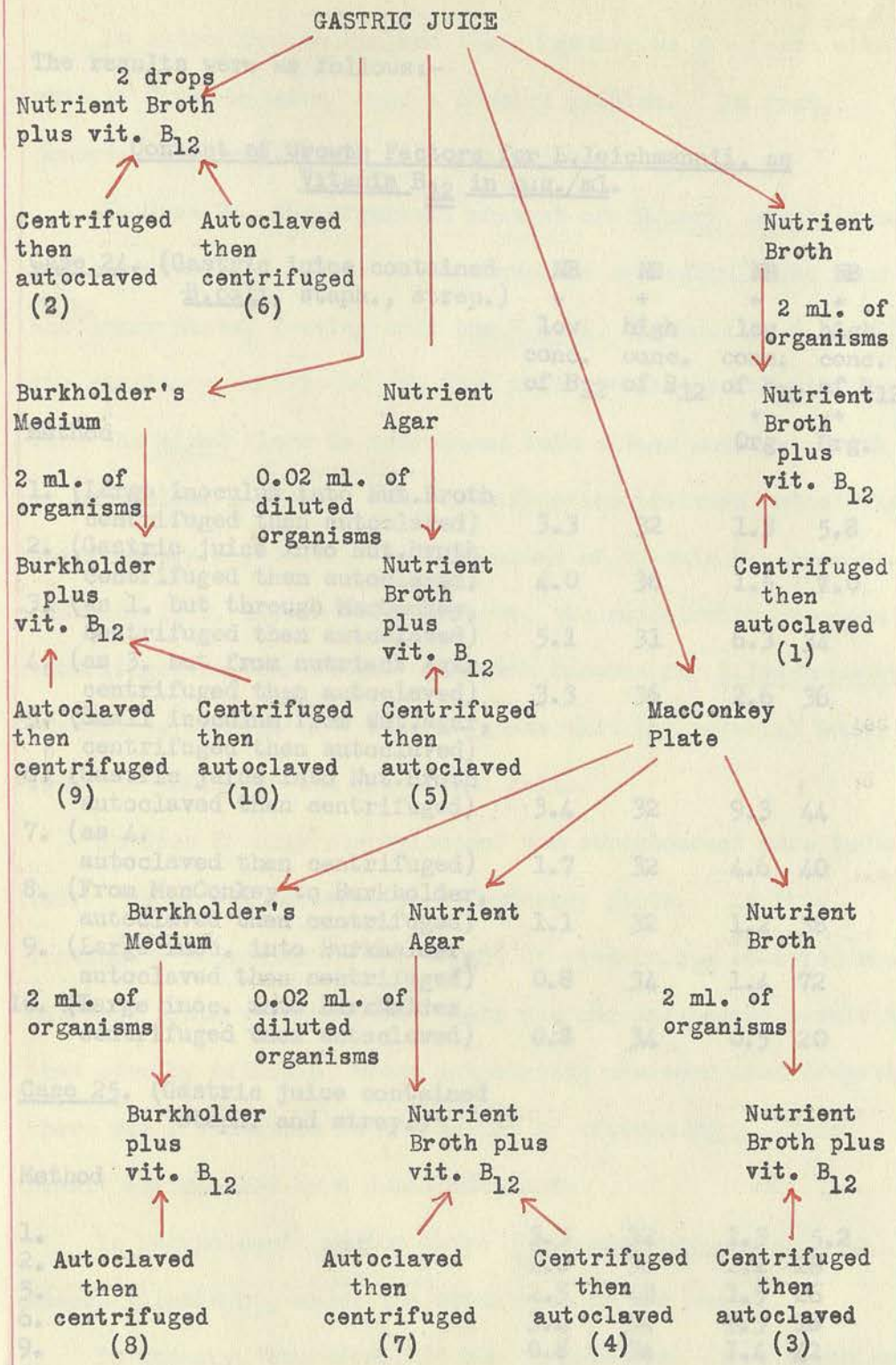
In/

In these instances the organisms were behaving as formerly.

INVESTIGATION No.6.

As a final investigation concerned with the vexed question of the effect of organisms on vitamin B_{12} under conditions of culture, gastric juice was obtained from two further cases of untreated pernicious anaemia (Cases 24 & 25 of this series).

From the gastric juice of Case 24 there were isolated B.coli, staphylococci and streptococci, and from Case 25 there were isolated only staphylococci and streptococci. The objects were (1) to apply to the organisms from the same patients the different techniques employed in the preliminary investigations and in Investigation No.1, and (2) to see whether any different results were found if the organisms in culture medium were autoclaved before centrifugation or alternatively centrifuged before autoclaving prior to the vitamin B_{12} assay being performed. (See pp.215 & 229.) This last might be an important variation in technique because the growth factors for L.leichmannii that were produced in the preliminary investigation might well have been released from the bodies of the organisms by the autoclaving. The various methods employed are shown diagramatically on p.245a. (It will be seen that a number is given to each method used.)


GASTRIC JUICE
2 drops Nutrient Broth plus vit. B_{12}
Centrifuged then autoclaved (2)
Autoclaved then centrifuged (6)
Nutrient Broth
2 ml. of organisms
Nutrient Broth plus vit. B_{12}
Centrifuged then autoclaved (1)
Burkholder's Medium
2 ml. of organisms
Burkholder plus vit. B_{12}
Autoclaved then centrifuged (9)
Centrifuged then autoclaved (10)
Nutrient Agar
0.02 ml. of diluted organisms
Nutrient Broth plus vit. B_{12}
Centrifuged then autoclaved (5)
MacConkey Plate
Burkholder's Medium
2 ml. of organisms
Burkholder plus vit. B_{12}
Autoclaved then centrifuged (8)
Nutrient Agar
0.02 ml. of diluted organisms
Nutrient Broth plus vit. B_{12}
Autoclaved then centrifuged (7)
Centrifuged then autoclaved (4)
Nutrient Broth
2 ml. of organisms
Nutrient Broth plus vit. B_{12}
Centrifuged then autoclaved (3)

The results were as follows:-

Content of Growth Factors for L.leichmannii, as Vitamin B_{12} in mμg./ml.

Case 24. (Gastric juice contained B.coli, staph., strep.) Method	NB + low conc. of B_{12}	NB + high conc. of B_{12}	NB + low conc. of B_{12} + Org.	NB + high conc. of B_{12} + Org.	Amount of B_{12} increased ↑ diminished ↓ or unchanged →
1. (Large inoculum into Nut.Broth centrifuged then autoclaved)	3.3	32	1.3	5.8	↓
2. (Gastric juice into Nut.Broth centrifuged then autoclaved)	4.0	36	1.5	7.0	↓
3. (as 1. but through MacConkey, centrifuged then autoclaved)	5.1	31	6.3	34	→
4. (as 3. but from nutrient agar, centrifuged then autoclaved)	3.3	36	2.6	36	→
5. (Small inoculum from Nut.agar, centrifuged then autoclaved)					
6. (Gastric juice into Nut.Broth autoclaved then centrifuged)	3.4	32	9.3	44	↑
7. (as 4. autoclaved then centrifuged)	1.7	32	4.6	40	↑
8. (From MacConkey to Burkholder, autoclaved then centrifuged)	1.1	32	1.2	38	→
9. (Large inoc. into Burkholder, autoclaved then centrifuged)	0.8	34	1.4	72	↑
10. (Large inoc. into Burkholder centrifuged then autoclaved)	0.8	34	0.5	20	↓
Case 25. (Gastric juice contained staph. and strep.)					
Method					
1.	3.3	32	1.3	5.2	↓
2.	4.0	36	2.2	28	↓
5.	2.5	40	1.5	26	↓
6.	3.4	32	3.3	16	↓
9.	0.8	34	1.4	42	↑
10.	0.8	34	0.4	34	→

NB = Nutrient Broth. Org. = Organisms.

In attempting to analyse these results we are faced with what at first appears to be a complex problem. In fact, however, the results are fairly consistent.

In Case 24, the organisms present are B.coli, staphylococci and streptococci. When these organisms are subcultured from a MacConkey plate, leaving only the B.coli, (Methods 3, 4, 7, 8), the strain appears to be one that does not absorb vitamin B_{12}. When the mixed flora is introduced into either nutrient broth or Burkholder's medium, and centrifugation precedes autoclaving, there occurs a reduction in the amount of vitamin B_{12} present. (Methods 1, 2, 10). When, however, the autoclaving precedes centrifugation, the amount of growth factors for L.leichmannii present is increased. This suggests that the material comes from the bodies of the organismal cells.

In Case 25 only staphylococci and streptococci were isolated. No growth occurred on a MacConkey plate. In this instance a reduction in the amount of vitamin B_{12} occurred where nutrient broth was used, but there was one unexpected result in that even by method 6, where autoclaving preceded centrifugation, there was a reduction in the amount of vitamin B_{12}. This result was checked by a duplicate assay.

In Burkholder's medium these organisms were not able to absorb vitamin B_{12} under the conditions of our assay.

In summary, therefore, it can be said that where organisms absorb vitamin B_{12}, this vitamin or some other substance which is a growth factor for L.leichmannii may be released if the culture in its medium is autoclaved prior to the centrifugation which/

which precedes the L.leichmannii assay. It was shown previously (p.222) that the substance produced, like vitamin B12, is destroyable by alkaline hydrolysis, but that it is not a growth factor for a mutant of B.coli. The possibilities are that the organisms, when autoclaved, release any absorbed vitamin B12, any substance into which they have converted it, and any other growth factors for L.leichmannii that they may have synthesised.

It was felt that little reward might be anticipated from following this matter further since the haemopoietic activities of any substances produced are unknown.

INVESTIGATION No.7.

This was a return to the investigation of the ability of various organisms to synthesise folic acid. After our preliminary investigations had been done there appeared a paper by Lascelles & Wood (1952) showing that certain strains of B.coli (those requiring p-aminobenzoate, and a normal strain) and of Staph.aureus (normal strain and a sulphathiazole resistant strain) were able to synthesise folic acid in a mixture containing buffer, glucose, glutamate and p-aminobenzoate. The latter was essential or stimulatory in every case. Sulphathiazole inhibited the synthesis. The sulphathiazole-resistant strain of Staph.aureus synthesised about ten times as much folic acid as the parent strain under similar conditions.

It has been shown that certain organisms such as Streptobacterium plantarum, Lactobacillus arabinosus and Clostridium acetobutylicum, which normally require p-AB as a growth factor, can grow when pteroylglutamic acid (PGA) is supplied instead, although the molar concentration necessary is 10 to 50 times higher. With all these organisms inhibition of growth by sulphonamides is overcome by both p-AB and by PGA. The organisms, however, are essentially insensitive to sulphonamide inhibition in the presence of PGA (see Woods, 1950; Nimmo-Smith & Woods, 1948; Lampen & Jones, 1947; Sims & Woods, 1949).

However it has been shown that pteroylglutamate behaves differently with other organisms requiring p-AB for growth. Thus Lampen et al., (1949) showed that it would not replace either the growth factor or anti-sulphonamide function of p-AB with/

with an induced mutant strain of B.coli. The studies of Lascelles & Woods (1952) were to see whether organisms like those for which PGA is inactive can synthesise folic acid from p-AB in simple systems.

We followed the methods of Lascelles & Woods (1952), but used in place of their parent strain of Bact.coli or X-ray induced mutant requiring p-AB, the organisms isolated from the alimentary tract that we have been considering in Investigations No.1 to No.6

METHODS

The organisms had been maintained by subculture in nutrient broth.

Assays of folic acid in the culture medium employed were done in the usual manner that we have already described, S.faecalis being used as the test organism.

Media used.

Medium A.

Glucose	2 G.
l-asparagine	2 G.
$(NH_4)_2 SO_4$	5 G.
Na Cl	5 G.
$K H_2PO_4$	3 G.
$Mg SO_4 7H_2O$	2 mg.
$Ca Cl_2 6H_2O$	2 mg.
*Iron-citrate solution	3 ml.
Water to	1,000

* 236 mg. sodium citrate ($3H_2O$) and 168 mg.Fe SO_4 $(NH_4)_2 SO_4 6H_2O$ dissolved in 100 ml. H_2O.

Medium B.

Medium A with the addition of

2.5×10^{-8} M p - AB

Medium C.

Medium A with the addition of

10/

10^{-6}M sulphathiazole.

Medium D.

p-AB (10^{-5}M)
Glucose (0.01 M)
l-glutamate (0.01 M)
*Phosphate buffer at pH 6.8 (0.1 M)

*Prepared from $Na_2HPO_4 . 12H_2O$ and KH_2PO_4.

The procedure employed was as follows:

The medium A (25 ml.) was inoculated with 0.02 ml. of organisms from nutrient broth and incubated for 14 hours at 37° C. The cells were centrifuged out and washed once with the culture volume of 0.02 M phosphate buffer, pH 6.8.

In order to reduce the amount of folic acid that might be present, the organisms were then grown in medium B or Medium C for 14 hours at 37° C.

The cells were finally suspended to a concentration of about 0.2 mg. dry weight per ml. in medium D, and incubated for 5 hours at 37° C. The suspensions were then diluted with an equal volume of water and autoclaved for 10 minutes at 10 lbs. pressure. The supernatant fluid after the centrifugation, which was done after autoclaving, was used for the S.faecalis assay.

The results obtained were as follows:

Organisms passed through medium B to deplete them of folic acid, then grown in medium D.

Source		
Case 12	Pernicious anaemia.	B.coli, intermediate II
13	Pernicious anaemia	B.aerogenes I, Staphylococcus

15/

Case 15	Regional jejunitis	B.aerogenes I, Staphylococcus, Streptococcus
18	Idiopathic steatorrhoea	B.coli Type I, Staphylococcus
19	Peripheral neuritis	Staphylococcus, Streptococcus
20	Macrocytic anaemia	B.coli Type I
23	Ileo-sigmoid fistula	B.coli, intermediate I.

No synthesis of folic acid occurred in any instance.

Organisms passed through medium C to deplete them of folic acid, then grown in medium D.

Source

Cases 12, 15, 19, 20 and 23.

No synthesis of folic acid occurred in any instance.

It may therefore be said that under the conditions of our investigations these organisms were unable to synthesise folic acid even in the presence of p-AB.

DISCUSSION

It is felt that a lengthy discussion should be avoided here. Our various investigations appear to show that many organisms may, under certain experimental conditions, be shown to produce a growth factor for L.leichmannii which may be pseudovitamin B_{12}. It is possible however that this was liberated by manipulation from the dead bodies of bacteria. Under different experimental conditions certain organisms may absorb vitamin B_{12}. Certain strains of B.coli and a mixture of organisms seemed able to synthesise a growth factor for S.faecalis, while others could not do so. Under other experimental conditions none of the organisms tested could synthesise such a factor. Moreover, after progressive subculturing, the mixed flora from one patient's gastric contents lost its capacity to absorb vitamin B_{12}, but when it was passed through a vitamin B_{12} deficient medium, this capacity was regained.

A study of these organisms and of their ability to produce, absorb, or modify haemopoietic factors under various conditions might well be the work of a lifetime, and the information might even then be of little value, because once the flora has been removed from the alimentary tract it is no longer possible to say that the results obtained are necessarily an indication of what takes place in the alimentary tract. What occurs in the stomach or in the large intestine is not likely to affect materially the intake of haemopoietic factors, and what occurs in/

in the small intestine is difficult to assess.

In relation to this it is of interest that recently Cregan & Hayward (1953) and Cregan et al. (1953) have examined the flora in the alimentary tract of twenty-two patients. They claim that when large numbers of bacteria enter a normal small intestine from a diseased stomach, they are destroyed by the time the mid gut is reached, if not earlier, and state that lowered gastric acidity, even achlorhydria, is not necessarily accompanied by an increase of bacteria in the stomach. The chief organisms these workers found in the upper intestine were predominantly Gram-positive species of the type more commonly associated with the mouth than with the lower intestine. They state that advanced disease of the stomach with achlorhydria does not encourage a resident flora in the small intestine, and consider that an antibacterial mechanism, distinct from that of the stomach, must operate in the small intestine. According to these workers consideration of a single specimen from the upper small intestine is misleading. The upper jejunum may be contaminated by overflow from a stomach containing a resident flora, but even then there is a scarcity of organisms lower down the small intestine. There workers pour scorn on the view that vitamin deficiencies in the post-gastrectomy syndrome, sprue, pellagra, and nutritional macrocytic anaemia may arise because of bacteria invading the small intestine. However, they do not include cases of the above conditions in their own investigations. Recently Howie et al. (1953) have not only shown Clostridium welchii to be present in the stomach contents during the first week after the operation of partial gastrectomy/

gastrectomy, but have shown small amounts of welchii α toxin to be present there also. These workers did not, however, investigate the bacteriology of the small intestine. It should be noted that Davidson (1928) quotes Van des Reis (1925) and Seyderhelm (1922, 1928) as finding that the sepsis which occurs in the gastro-duodenal region in pernicious anaemia continues throughout the whole length of the small intestine.

One final consideration that may be mentioned here is that certain compounds such as 5,6-Dimethyl-benziminazole which are structurally related to certain components of the vitamin B_{12} molecule can partially replace vitamin B_{12} in the nutrition of the rat. It has also been shown that these substances prevent the utilisation of vitamin B_{12} by organisms such as L.leichmannii and the vitamin B_{12}-requiring mutant strain of B.coli. It therefore seemed to be of interest to administer one of these substances by mouth to a pernicious anaemia patient together with a small amount of cyanocobalamin in order to see whether the benziminazole compound would prevent the hypothetical uptake of vitamin B_{12} by intestinal organisms. By the kind cooperation of Dr. W.F.J.Cuthbertson of Glaxo Laboratories Ltd. it was possible to obtain a small amount of 5,6-Dimethyl-benziminazole. Trial of this substance in one patient gave no haemopoietic response. The case is reported on p. 442.

SUMMARY/

SUMMARY

An account is given of an attempt to isolate haemopoietic factors from the alimentary tract of three fasting patients with pernicious anaemia and two controls. It was only in the faeces that any significant amounts were found.

A series of experiments were carried out with organisms isolated from the gastric contents of a number of patients with and without megaloblastic anaemia to see whether the organisms would, under conditions of culture, alone or in a mixture, produce, absorb, destroy or modify the haemopoietic substances vitamin B_{12}, folic acid and citrovorum factor. Under certain conditions it seemed that pseudovitamin B_{12} was produced by a large number of organisms, although it was possibly released from the dead bodies of the organisms. Under other conditions vitamin B_{12} was absorbed by them, but it was not possible to show that gastric juice containing intrinsic factor prevented such absorption. A few strains of organisms appeared to produce folic acid under the conditions of the investigation.

It is, however, concluded that although these investigations are of some interest, caution must be exercised in reaching any conclusions from them about what takes place with a mixture of organisms under the very different conditions occurring in the alimentary tract.

CHAPTER 6

THE CONTENT OF HAEMOPOIETIC FACTORS IN THE ORGANS AND TISSUES OF MAN

The first report on the content of vitamin B_{12} in various organs and tissues of animals was by Lewis et al. (1949), who used a rat growth method of assay. It is difficult to be sure about what is being measured by this type of assay, and more reliable are the figures of Couch and Olcese (1950) who used a L.leichmannii assay to measure the vitamin B_{12} content of the organs of chicks. These workers found that when the diet fed contained all vegetable protein, the content of vitamin B_{12} in the various organs, measured in µg./100 G. were liver 3.8, kidney 4.6, pancreas 5.7 and spleen 10.8. When there was added to the diet 2 µg. of vitamin B_{12} per 100 G. of feed, the figures for vitamin B_{12} content were liver 18.6, kidney 8.3, pancreas 7.3 and spleen 12.4 µg./100 G. This showed that the composition of the diet may affect the tissue stores of vitamin B_{12}. When there was added to the diet 10% of dehydrated alfalfa meal in place of an equivalent amount of yellow corn, there appeared to be some inhibitory effect, since the content of vitamin B_{12} fell, the figures then being liver 2.9, kidney 3.2, pancreas 3.4 and spleen 5.2 µg./100 G. All the figures represent the mean of several estimations on different chicks. Whether alfalfa contains some form of vitamin B_{12} antagonist has not been investigated.

More recently Yacowitz et al. (1952), also using a L.leichmannii /

L.leichmannii assay and employing chicks fed a diet containing 50 μg. of vitamin B_{12} per 100 G. of feed obtained the following mean figures (in μg./100 G.) liver 44.4, kidney 110.7, pancreas 42.5, spleen 15.5, heart 20.1.

Surprisingly there were no reports of the content of haemopoietic factors in the tissues and organs of man when the present investigation was commenced.

The methods employed were described in Chapter 4.

EXPERIMENTAL.

Various tissues and organs were obtained at autopsy from the following patients.

Case I. A man aged 65, was admitted to hospital with severe untreated pernicious anaemia diagnosed on the clinical and haematological findings which included sternal puncture. The haemoglobin level was 5.7 G./100 ml., R.B.C. 1.95 millions/cu.mm. Before treatment commenced the patient died suddenly of a coronary thrombosis.

Case 2. A woman aged 79, was admitted to hospital with untreated pernicious anaemia (Hb. 5.3 G./100 ml., R.B.C. 1.35 millions/cu.mm.). She was given 2 ml. of liver extract on admission (containing about 6 μg. of vitamin B_{12}/ml.) and the following day 100 μg. of vitamin B_{12} together with a transfusion of a pint of blood. She died on the third day in hospital through having aspirated her vomitus into the trachea and bronchi. At the time of death the marrow was not completely converted to the normoblastic state.

Case 3. A woman aged 57 was admitted to hospital with hypertensive cardiac failure. There was collapse of the left lung and at autopsy a bronchial carcinoma was found to be present. No visible tumour deposits were present in the tissues that were used for assay. The patient was not anaemic.

Case 4. A man aged 75 was admitted to hospital with a cerebral thrombosis and died the following day. There was no anaemia.

Cases 5, 6, 7 and 8 were non-anaemic patients who were undergoing partial gastrectomy operations for gastric ulcer./

ulcer. By the kindness of Mr. A. Lowdon and Mr. T. Miller of the Royal Infirmary of Edinburgh, pieces of liver weighing about 2 G. were obtained at operation.

RESULTS.

Significance of assay procedures.

Nichol & Welch (1950) have shown that pteroylglutamic acid may be converted to citrovorum factor in tissues under certain conditions, and hence we have followed the practice of Swendseid et al. (1951) of adding pteroylglutamic acid to samples of certain of the tissues prior to incubation to see whether such conversion occurred under the conditions of our assay. When pteroylglutamic acid had been added to the fluid in which the tissues were homogenised, there was no increase in the ability of samples to stimulate the growth of L.citrovorum although the added pteroylglutamic acid could be recovered to the extent of 80-98%, as shown by the Strep. faecalis assay (Table 34). When citrovorum factor was added before homogenisation, recoveries were equally satisfactory, and hence there was no evidence of conversion of pteroylglutamic acid to citrovorum factor or citrovorum factor to pteroylglutamic acid.

In all instances, assays were performed at least in duplicate, usually in quadruplicate, and the accuracy of the method was checked by performing repeated assays on several specimens of the same tissue, separately weighed and homogenised (Table 35).

Content of vitamin B_{12} and of growth factors for Streptococcus faecalis in a patient with untreated pernicious anaemia.

The results of this investigation are given in Table 36. It will be seen that no vitamin B_{12} was detected in any organs

TABLE 34

RECOVERY OF PTEROYLGLUTAMIC ACID AND CITROVORUM FACTOR ADDED TO THE BUFFER IN WHICH HUMAN TISSUES WERE HOMOGENISED

Organ	Method of extraction	Supplement added to homogenate	CF activity (L.citrovorum assay) (μg./G.)	PGA acid activity (S.faecalis assay) (μg./G.)
Liver	B	None	0.67	0.61
	B	10 μg. PGA/G.	0.68	9.8
	B	4.38 μg. CF/G.	4.3	4.1
	A	None	0.45	0.47
	A	10 μg. PGA/G.	0.44	9.0
	A	4.38 μg. CF/G.	4.1	3.9
Liver	B	None	1.0	0.79
	B	0.922 μg. CF/G.	1.8	1.4
Kidney	B	None	0.22	0.10
	B	0.8 μg. PGA/G.	0.25	0.89
	B	0.922 μg. CF/G.	0.97	0.73

TABLE 35

RESULTS OF REPEATED ASSAYS ON DIFFERENT SPECIMENS OF SAME ORGANS

Case	Organ	Method of estimation	Vit.B_{12} content (L.leichmannii assay) (µg./100 G.)	Growth factors for S.faecalis (µg./100 G.)	CF content (L.citrovorum assay) (µg./100 G.)
3	Liver	B	22, 29, 25	120, 130, 120	150, 160, 180
		A	23, 26, 24	130, 140	150, 150
4	Liver	B	58, 53, 50	390, 410	530, 520

TABLE 36

VITAMIN B_{12} AND TOTAL APPARENT PTEROYLGLUTAMIC ACID CONTENT OF THE TISSUES AND ORGANS OF A PATIENT WITH UNTREATED PERNICIOUS ANAEMIA CASE 1

(The vitamin B_{12} content (*L.leichmannii* assay) without cyanide was nil in all these.)

Organ or tissue	Method of extraction	Total apparent folic acid activity (*S.faecalis* assay) (µg./100 G.)
Liver	B	83
	A	90
Kidney	B	24
	A	5.5
Spleen	B	14
	A	8.9
Lung	B	Negligible
	A	Negligible
Brain	B	5.4
	A	-
Stomach	B	Negligible
	A	Negligible
Intestine	B	14
	A	3.9
Tongue	B	12
	A	3.4
Muscle	B	8.2
	A	4.1

or tissues. In the assay method employed each tube contained 10 ml. of fluid of which 5 ml. comprised double strength medium. When 0.2 mμg. of vitamin B_{12} was added to a series of such assay tubes containing 0.5, 1, 2, 3 and 4 ml. respectively of extracts of each of the tissues of this patient, prepared as in method B, but brought to a final dilution of 100 ml., growth occurred equally in all tubes except in the case of the extract of skeletal muscle where it was progressively less with increasing content of tissue extract. Since the amount of growth in all other instances was equal to that produced by 0.2 mμg. of vitamin B_{12} in water, it appears that in most of the tissues no inhibitors were present to prevent the growth-stimulating effect of vitamin B_{12} for L.leichmannii.

Content of vitamin B_{12}, pteroylglutamic acid and citrovorum factor in the organs and tissues of control patients.

The results of this investigation are given in Table 37. It will be seen that in the liver and kidney almost all the apparent pteroylglutamic acid was, in fact, citrovorum factor, but that in most instances the other organs and tissues contained both of these forms. In a patient who died of cirrhosis of the liver* the content of vitamin B_{12} in the organ was 41 μg./100 G. and the folic acid activity was 244 μg./100 G. No therapy with antimegaloblastic substances had been given.

Content of haemopoietic factors in the organs and tissues of a pernicious anaemia patient at the commencement of treatment.

The results of this investigation are given in Table 38. It will be seen that here, in contrast to what was found in cases/

* See also p.628, Chapter 17.

TABLE 37.

CONTENT OF HAEMOPOIETIC FACTORS IN THE TISSUES OF NON-ANAEMIC PATIENTS.

Case no.	Organ or tissue	Method of extraction	Vitamin B_{12} content (*Lb. leichmannii* assay) Without cyanide (μg./100 g.)	Vitamin B_{12} content (*Lb. leichmannii* assay) After cyanide treatment (μg./100 g.)	Total apparent folic acid activity (FAA) *Strep. faecalis* assay (μg./100 g.)	Apparent citrovorum-factor activity (ACF) *Strep. faecalis* assay (μg./100 g.)	True citrovorum-factor activity (CF) *L. citrovorum* assay (μg./100 g.)	True folic acid activity (PGA) (μg./100 g.)
3	Liver	B	25	28	130	170	160	Negl.
		A	24	28	138	160	150	Negl.
	Kidney	B	16	15	79	84	90	Nil.
		A	7.1	7.1	16	16	14	Negl.
	Spleen	B	3.9	4.7	39	47	47	Nil
		A	4.6	5.0	6.0	7.8	9.9	Nil
	Lung	B	5.1	5.2	65	76	42	30
		A	4.0	4.2	33	36	9.4	26
	Brain	B	9.1	10	38	48	14	27
		A	7.0	8.7	12	18	1.7	11
	Muscle	B	8.3	8.4	39	42	24	21
		A	7.8	6.4	7.5	13	3.9	4.5
	Skin	B	1.3	1.5	24	25	7.8	18
		A	1.4	1.2	15	17	Negl.	15
4	Liver	B	54	67	400	520	520	Nil
		A	55	74	400	510	540	Nil
	Kidney	B	25	32	38	38	40	Nil
		A	33	37	34	41	42	Nil
	Spleen	B	36	39	71	78	29	49
		A	12	21	16	19	5.8	11
	Brain	B	22	22	17	20	3.7	14
		A	5.9	6.1	Negl.	Negl.	Negl.	Negl.
	Stomach	B	22	30	38	40	23	21
		A	6.2	7.8	21	20	8.1	15
	Intestine	B	28	27	23	26	3.1	20
		A	15	16	20	20	5.3	16
	Muscle	B	11	16	18	20	17	5.4
		A	9.9	12	16	13	9.1	9.7
5	Liver	B	50	-	430	550	500	41
		A	46	-	310	390	300	81
6	Liver	B	53	-	270	350	340	52
7	Liver	B	153	-	410	640	590	32
		A	110	-	380	520	510	54
8	Liver	B	68	-	220	330	280	31

TABLE 38.

CONTENT OF HAEMOPOIETIC FACTORS IN THE ORGANS AND TISSUES OF A PERNICIOUS ANAEMIA PATIENT AT COMMENCEMENT OF TREATMENT. CASE 2.

Organ	Method of extraction	Vitamin B_{12} content (*Lb. lechmannii* assay) Without cyanide (μg./100 g.)	Vitamin B_{12} content (*Lb. lechmannii* assay) After cyanide treatment (μg./100 g.)	Total apparent folic acid activity (FAA) *Strep. faecalis* assay (μg./100 g.)	Apparent citrovorum-factor activity (ACF) *Strep. faecalis* assay (μg./100 g.)	True citrovorum-factor activity (CF) *L. citrovorum* assay (μg./100 g.)	True folic acid activity (PGA) (μg./100 g.)
Liver	B	14	23	120	140	79	62
	A	3.2	4.1	65	66	26	45
Kidney	B	5.0	7.3	46	43	46	Nil
	A	2.6	2.9	11	15	12	2.0
Skin	B	0.27	-	11	16	1.5	9.7
	A	0.26	Negl.	Negl.	Negl.	Negl.	Negl.

cases 3 and 4, an appreciable part of the apparent pteroylglutamic acid content was not due to citrovorum factor. The patient received approximately 112 μg. of vitamin B_{12} by injection before death. The liver weighted 1120 G. and the kidneys each weighed 120 G. If the highest figure obtained for B_{12} content (method B, treated with sodium cyanide) is taken to be the correct one, then the liver contained 260 μg. of vitamin B_{12} and in the kidneys there was a further 17.8 μg.

DISCUSSION.

These results given an indication of the occurrence of vitamin B_{12}, pteroylglutamic acid and citrovorum factor in human tissues and indicate that in the liver and kidneys the apparent pteroylglutamic acid is largely citrovorum factor. It is possible, but unlikely, that this was due to post-mortem conversion of pteroylglutamic acid to citrovorum factor because (1) when pteroylglutamic acid was incubated with liver or kidney, which did not appear themselves to contain appreciable amounts of this substance, conversion to citrovorum factor did not occur; (2) similar results were found by Swendseid et al. using fresh mouse livers; (3) in the liver obtained at surgical operation, growth of S.faecalis was very largely due to citrovorum factor; and (4) in the liver of the pernicious anaemia patient (case 2) there was no increase of the percentage of growth activity for S.faecalis, due to citrovorum factor, after the organ had been kept frozen for 4 months. Unfortunately, it was not possible to carry out assays for citrovorum factor at the time when the tissues of case 1 were investigated, since at that time citrovorum factor for/

for use as a standard was not available.

Although the terms pteroylglutamic acid and citrovorum factor have been used above, this is probably an over-simplification, since it may well be that the method of preparation of the organs for assay converts conjugates of pteroylglutamic acid or citrovorum factor into the 'free' forms. To illustrate the difficulties of coming to any conclusion, the following two small investigations may be described. The first was designed in the first instance to see whether citrovorum factor still predominated when overnight incubation was not included in the preparation of the homogenate.

(1) A watery extract was made of the liver of case 7. No incubation with buffer was carried out, an immediate assay being made of this watery extract for its content of growth factors for S.faecalis and L.citrovorum. This was done in triplicate. The results were:

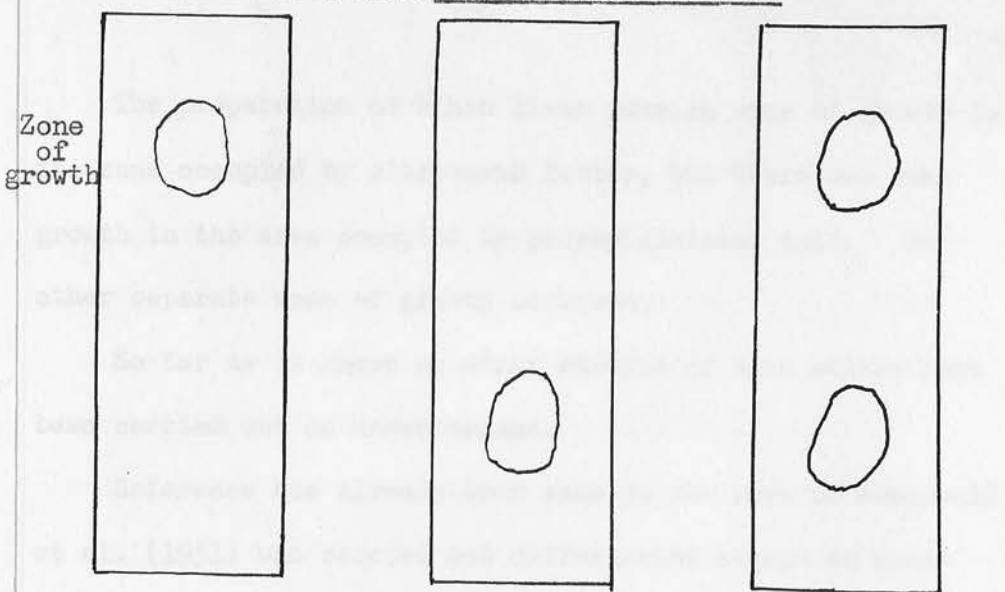
Total apparent folic acid activity (S.faecalis assay)	8.0 µg./100 G.
Apparent citrovorum factor activity (S.faecalis assay)	12.5 µg./100 G.
True citrovorum factor activity (L.citrovorum assay)	11.4 µg./100 G.
True pteroylglutamic acid content	1.2 µg./100 G.

This small experiment again suggests that most of the growth activity for S.faecalis is due to citrovorum factor but that some pteroylglutamic acid may be present, probably in an unconjugated form, since the conjugate pteroylhexaglutamylglutamic acid does not support the growth of S.faecalis. The difficulty about making any similar conclusion in relation to citrovorum factor is that although the occurrence of citrovorum factor/

factor conjugates is suspected they have not been identified, so it is not known whether or not they themselves would support the growth of S.faecalis, L.citrovorum, or both, or neither.

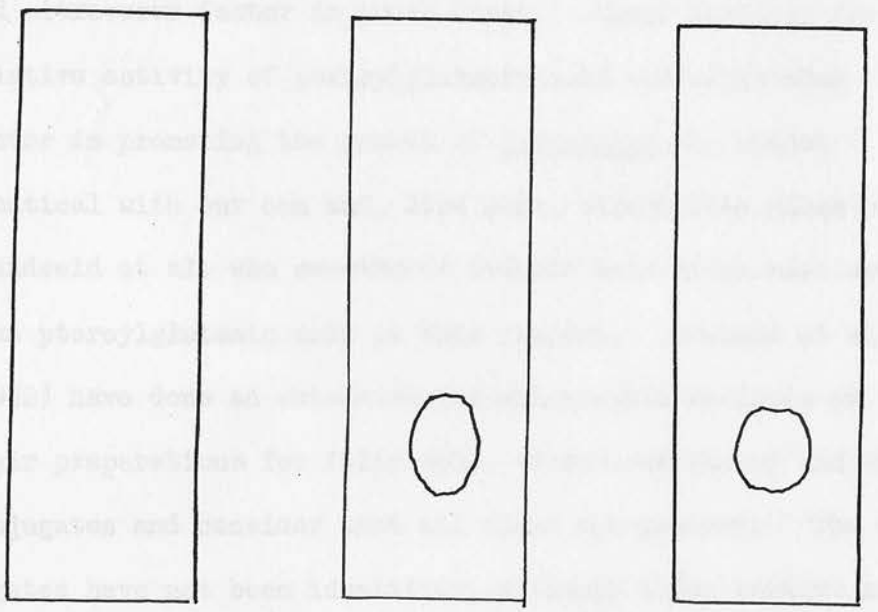
(2) A second method of approach involved the use of a paper chromatographic technique, and was a modification of the method used by Winsten & Eigen (1944) for the separation of vitamin B_{12} like substances and demonstrated by them to the author when he was in New York. Strips of Whatman's filter paper No.2 were used. The method employed a two-phase solvent system with a layer of 5% disodium phosphate and a thinner layer of butyl alcohol. The paper dipped into the solvent system contained in a glass trough at the top of the jar. A small amount of liver homogenate prepared from a specimen obtained at surgical operation and treated by incubation with buffer without pancreatin as described in Chapter 4 was put on a pencilled starting line. After some 18 hours the strips were removed from the solvent system and dried. Control strips had synthetic pteroylglutamic acid or citrovorum factor or a mixture of these applied to them. Similar strips were put on agar plates seeded with L.citrovorum or S.faecalis, and were then incubated for 16 hours. The zones of growth were easily seen, and from their position it was possible to identify growth due to citrovorum factor, pteroylglutamic acid, or other unknown substances. The results were of the following nature:

ASSAY WITH STREPTOCOCCUS FAECALIS



Pteroylglutamic acid applied. | Citrovorum factor applied. | Pteroylglutamic acid & Citrovorum factor applied.

ASSAY WITH LEUCONOSTOC CITROVORUM



Pteroylglutamic acid applied. | Citrovorum factor applied. | Pteroylglutamic acid & Citrovorum factor applied.

Fig. 6. Separation by paper chromatography of pteroylglutamic acid and citrovorum factor.

The preparation of human liver gave an area of growth in the zone occupied by citrovorum factor, but there was some growth in the area occupied by pteroylglutamic acid. No other separate zone of growth occurred.

So far as is known no other studies of this nature have been carried out on human organs.

Reference has already been made to the work of Swendseid et al. (1951) who carried out differential assays on mouse liver homogenates, and considered that most of the folic acid activity was due to citrovorum factor. Since our own studies were completed, Wieland et al. (1952) have also published a paper dealing with the measurement of the ratio of folic acid and citrovorum factor in mouse liver. Their findings for the relative activity of pteroylglutamic acid and citrovorum factor in promoting the growth of S.faecalis are almost identical with our own and, like ours, differ from those of Swendseid et al. who considered folinic acid to be more active than pteroylglutamic acid in this respect. Wieland et al. (1952) have done an extensive chromatographic analysis of their preparations for folic acid, citrovorum factor and their conjugates and consider that all these are present. The conjugates have not been identified, although these workers used for their bioautographic controls the substances pteroic acid, pteroylglutamyl-α-glutamic acid, pteroylglutamyl-γ-glutamic acid, pteroylglutamyl-γ-glutamyl-γ-glutamic acid, thymine, thymidine, N^{10} Formylfolic acid, and an enzymatic digest of desoxyribonucleic acid.

Our/

Our conclusion from our own investigations is that most of the folic acid activity in the normal human liver is due to citrovorum factor but that it is not possible to say to what extent it is due to citrovorum factor conjugates. It does seem that there is also some folic acid possibly in a free form, but the fact that chromatographs do not show zones of growth in areas distinct from those occupied by pteroyl-glutamic acid or citrovorum factor does not exclude the possibility that some of the folic acid is in an unknown conjugate. form. The technique of preparation of homogenates is such that it cannot be said with certainty that a method involving no enzymatic treatment of the organs releases only 'free' folic acid and 'free' citrovorum factor.

The liver of case 2, a pernicious anaemia patient, at the commencement of treatment, differs from that of the other patients in that there was as much 'folic acid' as there was citrovorum factor. This might mean that the pernicious anaemia patient is unable to convert pteroylglutamic acid to citrovorum factor, but on the other hand case 2 did not have more folic acid in the liver than did some of the control cases, so that this may merely indicate that the liver of the pernicious anaemia patient is deficient in citrovorum factor.

It will be noted that in common with other workers we found that with certain tissues the content of haemopoietic factors was greater when pancreatin extraction was used, and there is no reason to believe that the higher figure in each instance is not the correct one. In most instances the addition/

addition of sodium cyanide after homogenisation caused little increase in the content of vitamin B_{12} suggesting that little vitamin B_{12b} was present, though Cuthbertson (1951) states that cyanide is more effective if added prior to homogenisation.

No vitamin B_{12} was found in the tissues and organs of case 1, and except with the extract of muscle there was no evidence for the presence of any inhibitors that would prevent added vitamin B_{12} from supporting the growth of the test organism. The findings for the vitamin B_{12} content of the organs in case 2 are more difficult to interpret. It is known that when vitamin B_{12} is given by injection in doses of 100 µg. or more, there is a considerable and rapid excretion of much of the vitamin in the urine, and yet the combined vitamin B_{12} contents of the liver and kidneys appear to be greater than the amount that was injected. There is already some evidence that the tissues and organs of patients with pernicious anaemia in relapse are not always completely devoid of vitamin B_{12} (Girdwood, 1951; Drouett, Wolff & Rauber, 1951), but if this is the true explanation it would appear reasonable to assume that the vitamin B_{12} present in the tissues of such patient is in a 'bound' form that is not available for haemopoiesis. An alternative interpretation would be that some other substance is present that stimulates the growth of L.leichmannii under the conditions of the assay despite the use of a technique that includes alkaline hydrolysis.

If we take the weight of a human liver to be 1,000 G., and/

and its normal content of vitamin B_{12} to be 50 μg./100 G., the injected dose of vitamin B_{12} required to saturate the liver (quite apart from the other organs and tissues) is 500 μg. However the pernicious anaemia patient, like the normal person rapidly excretes a high proportion of an injected dose, so that it appears that the kidney excretes vitamin B_{12} more rapidly than it can be 'fixed' in the tissues.

SUMMARY.

For the first time an estimation has been made of the content of haemopoietic factors in human organs and tissues. The highest content of vitamin B_{12} is found in the liver and kidney in normal persons. In an untreated pernicious anaemia patient no vitamin B_{12} was found in any organ, whereas in a second patient who had died at the commencement of treatment, some vitamin B_{12} (and probably B_{12b}) was recovered from the liver and kidney. In the normal liver most of the folic acid activity was due to citrovorum factor but there was evidence for the presence of a much smaller amount of folic acid. It was not possible to be certain to what extent these were present as unidentified conjugates. In the pernicious anaemia patient who died at the commencement of treatment there were about equal amounts of folic acid and citrovorum factor in the liver. This may have been due to an inability of the body to metabolise folic acid to citrovorum factor or to a normal amount of folic acid being present with a diminution in the content of citrovorum factor.

CHAPTER 7

SOME OBSERVATIONS ON THE OCCURRENCE OF GROWTH FACTORS FOR L.leichmannii, S.faecalis and L.citrovorum IN THE SERUM AND URINE OF CONTROLS AND OF PATIENTS WITH MEGALOBLASTIC ANAEMIA BEFORE TREATMENT AND AFTER TREATMENT WITH CYANOCOBALAMIN, PTEROYLGLUTAMIC ACID, PTEROYLDIGLUTAMIC ACID, PTEROYLTRIGLUTAMIC ACID AND CITROVORUM FACTOR

The main purposes of this part of the investigation were to see (1) whether any difference could be shown between the serum or urinary levels of vitamin B_{12}, folic acid and citrovorum factor in pernicious anaemia patients and various controls, (2) whether the administration of cyanocobalamin, pteroylglutamic acid or citrovorum factor affected the serum levels of the other two, (3) whether the synthetic folic acid conjugates were converted into folic acid (or citrovorum factor) in pernicious anaemia, (4) to what extent there was a difference in the urinary output of vitamin B_{12}, folic acid or citrovorum factor following the administration of these to pernicious anaemia patients and to controls.

This was done partly to assist in consideration of the metabolic alterations in pernicious anaemia and partly to see whether any test of diagnostic importance could be developed.

MATERIALS.

Serum. Blood was obtained by venepuncture in a dry syringe and was allowed to clot in a sterile glass container. The serum was removed and kept frozen until assayed; this was usually done within four days. No treatment with enzymes was required.

Urine. Twenty-four hour collections were made in brown bottles/

bottles containing a phosphate buffer and toluene. The specimens were stored at 4°C. and assays were usually done within four days.

METHODS.

The assay methods have already been described.

Recovery experiments performed on the urine and serum gave satisfactory results, as shown in Tables 39 and 40. It will be seen, however, from these tables and from the pages that follow that when quantities of less than 5 mμg. of cyanocobalamin were added to 1 ml. of serum the vitamin B_{12} would not be recovered unless the mixture was thereafter diluted and heated at 100°C. for 30 minutes. This was true both in a pernicious anaemia patient and a control.

The details of the method of carrying out recovery experiments with cyanocobalamin in relation to serum assays are complex and require to be given to facilitate understanding of Table 40.

Two standard cyanocobalamin solutions were made up. One contained 0.5 mμg./ml.: let it be called A.

The second contained 5 mμg./ml.: let it be called B.

(1) Take 3 ml. of serum.
Add 3 ml. of A.
Let this stand for 1 hour. It contains 1.5 mμg. of added cyanocobalamin in 6 ml.
Dilute this 1→2.
That equals 0.125 mμg./ml.
Add it to the medium in volumes of 1, 2, 3 and 5 ml. for assaying.

(2) Take 3 ml. of serum.
Add 1 ml. of B.
Let this stand for 1 hour. It contains 5 mμg. of added cyanocobalamin in 4 ml.
Dilute this 1→2. (dilution 2a).
That equals 0.625 mμg./ml.
Add it to the medium in volumes of 1, 2, 3 and 5 ml. for assaying.
Also dilute 2a 1→5 and use this for assaying (dilution 2b).

(3)/

(3) Take 3 ml. of serum.
Add 3 ml. of B.
Let this stand for 1 hour. It contains 15 mμg. of added cyanocobalamin in 6 ml.
Dilute this 1→2 (dilution 3a).
That equals 1.25 mμg./ml.
Add it to the medium in volumes of 1, 2, 3 and 5 ml. for assaying.
Also dilute 3 a 1→10 and use this for assaying (dilution 3b).

(4) Take 3 ml. of serum.
Add 6 ml. of A.
Let this stand for 1 hour. It contains 3 mμg. of added cyanocobalamin in 9 ml.
Dilute this 1→2 (dilution 4a).
That equals 0.17 mμg./ml.
Steam for 30 mins. at 100°C. and add it to the medium in volumes of 1, 2, 3 and 5 ml. for assaying.
Also dilute 4a 1→2 for assaying (dilution 4b).

(5) Take 3 ml. of serum.
Add 3 ml. of B.
Let this stand for 1 hour. It contains 15 mμg. of added cyanocobalamin in 6 ml.
Dilute this 1→4 (dilution 5a).
That equals 0.625 mμg./ml.
Steam for 30 mins. at 100°C. and add it to the medium in volumes of 1, 2, 3 and 5 ml. for assaying.
Also dilute 5b 1→5 for assaying (dilution 5b).

THE ESTIMATION OF HAEMOPOIETIC SUBSTANCES IN THE SERUM

Vitamin B_{12}.

Mollin & Ross (1952) have estimated the vitamin B_{12} content of the sera and urines of pernicious anaemia patients and controls, using *Euglena gracilis* as the test organism. They found that the total vitamin B_{12} concentration in the sera of normal subjects did not exceed 0.72 mμg./ml., and in patients with pernicious anaemia in relapse it did not exceed 0.1 mμg./ml. These figures apply to serum that had been heated at 100°C. for 15 minutes to release "free vitamin B_{12}" from its "combined form".

Attempts/

TABLE 39.

Recovery experiments performed on Urines to which Haemopoietic Factors had been added.

	Substance	Amount added to 1 litre of urine (mg.)	Amount recovered (mg.)
Normal Urine	Pteroylglutamic acid	5	4.7
		10	10.2
		15	14.3
	Citrovorum factor*	12	11.2
PA Urine	Pteroylglutamic acid	10	9.8

		Amount added to 1 litre of urine (µg.)	Amount recovered (µg.)
Normal Urine	Cyanocobalamin	20	19.2
		100	102
		20	20.4
PA Urine		20	20.5
		100	97.6
		20	19.8

* This was the commercial preparation 'Leucovorin'.

TABLE 40.

Recovery Experiments performed on Sera to which Haemopoietic Factors had been added.

Normal Serum

Substance	Amount added to 2 ml. of serum (mμg.)	Amount recovered (mμg.)
Pteroylglutamic acid	10	8.7
	10	11.8
	10	9.8
Citrovorum Factor	16	16.4
	16	15.8
	16	19.2

Normal Serum

	Amount present in 1 ml. of diluted serum (see p.274) (mμg.)	Amount recovered (mμg.)	Actual amount of cyanocobalamin added to 1 ml. of serum before dilution (see p.274) mμg./ml.	Method
Cyanocobalamin				
Serum unsteamed	0.125	Nil	0.5	1
	0.625	0.057	1.67	2(a)
	0.125	0.017	1.67	2(b)
	1.25	1.02	5.0	3(a)
	0.125	0.12	5.0	3(b)
Serum steamed	0.17	0.20	1.0	4(a)
	0.085	0.13	1.0	4(b)
	0.625	0.61	5.0	5(a)
	0.125	0.16	5.0	5(b)
	Pernicious Anaemia Serum			
Serum unsteamed	0.125	Nil	0.5	1
	0.625	0.088	1.67	2(a)
	0.125	0.017	1.67	2(b)
	1.25	0.92	5.0	3(a)
	0.125	0.12	5.0	3(b)
Serum steamed	0.17	0.23	1.0	4(a)
	0.085	0.12	1.0	4(b)
	0.625	0.65	5.0	5(a)
	0.125	0.13	5.0	5(b)

(These results have been corrected for the amount of growth factor present in the serum itself.)

Attempts to demonstrate vitamin B_{12} in the serum using the less sensitive organism L.leichmannii have usually been unsuccessful. In the present investigation, however, the serum was heated at 100°C. for 30 minutes after dilution 1 in 10 with water. It was found that this manipulation enabled the serum of both pernicious anaemia patients and controls to support the growth of L.leichmannii as shown in Table 41. This growth factor for L.leichmannii did not, however, appear to be vitamin B_{12} since pooled sera of pernicious anaemia patients or controls that had been treated by heat in the manner described above still supported the growth of the test organism when they were subjected to alkaline hydrolysis.

TABLE 41

SERUM CONTENT OF GROWTH FACTORS FOR L.leichmannii

(expressed as vitamin B_{12})

	No. of patients	Unheated serum mμg./ml.	Serum heated for 30 mins. at 100°C. mμg./ml.	
			Range	Mean
Controls	10	Negl.* - 0.07	0.1-2.8	1.38
Untreated pernicious anaemia patients	20	Negl.**-0.06	1.2-4.8	2.45
Other megaloblastic anaemias	4	Negl.	0.7-2.5	1.58

* No growth in 1 case.
** No growth in 14 cases.

However, it will be seen from Chapter 8 that when the technique of serum vitamin B_{12} assay by L.leichmannii was altered to include preliminary precipitation of proteins it

was/

was found possible to show a difference between the levels in pernicious anaemia patients and controls, and the content of vitamin B_{12} in the serum then became of the same order of magnitude as that shown by the Euglena assay. The remarks that follow in the present chapter refer to measurements without preliminary protein precipitation. It should however be stressed that the assay was measuring turbidity due to growth of L.leichmannii. No protein precipitation occurred in the course of the assay itself to invalidate the results.

Eleven patient with pernicious anaemia and 5 controls were given cyanocobalamin by injection or by mouth, and the serum levels of folic acid, citrovorum factor and of growth factors for L.leichmannii were estimated at intervals. The results obtained with L.leichmannii are shown in Table 42.

The administration of cyanocobalamin did not lead to any consistent change in the serum level of folic acid or of citrovorum factor. It will be seen that the parenteral administration of cyanocobalamin resulted in a rise of the serum level of vitamin B_{12} for a few hours both in pernicious anaemia patients and controls. When the serum was heated at 100°C. for 30 minutes, however, the rise in the serum content of growth factors for L.leichmannii above the resting level was greater than in the unheated specimens. It is difficult to manipulate such small quantities of serum, but it appeared that this increase was due to growth factors that were destroyed by alkaline hydrolysis and which, in the light of present knowledge, should be assumed to represent vitamin B_{12}. Hence the unheated serum taken a few hours after the administration of the/

TABLE 42.

Serum Levels of Growth Factors for L.leichmannii after Administration of Vitamin B_{12}.

(mμg./ml. expressed as vitamin B_{12})

Case*	0	½ hr.	1	2	3	5	8	24hr.	Dose of Vit. B_{12} & route μg.
Pernicious anaemia patients (in relapse)									
1(a)	.02	3.8	-	-	Negl.	-	-	Negl.	50 IV
(b)	1.2	7.1	-	-	4.7	-	-	2.3	
2(a)	Negl.	2.7	-	-	-	Negl.	-	Negl.	50 IV
(b)	1.5	13.5	-	-	-	10.2	-	3.5	
3(a)	Negl.	1.6	-	-	0.3	-	-	Negl.	50 IV
(b)	2.6	5.5	-	-	4.3	-	-	5.2	
4(a)	Negl.	-	2.3	-	-	0.02	-	0.04	50 IV
(b)	2.4	-	9.0	-	-	4.4	-	4.2	
5(a)	Negl.	9.0	-	-	-	-	-	-	50 IV
(b)	2.2	14.0	-	-	-	-	-	-	
6(a)	0.06	5.0	3.4	0.6	-	-	-	-	50 IV
(b)	3.8	9.0	6.9	5.4	-	-	-	-	
7(a)	Negl.	3.2	3.2	2.1	0.7	0.5	Negl.	0.2	50 IM
(b)	2.5	10.5	13.2	6.0	5.0	4.8	4.2	6.0	
8(a)	Negl.	2.8	2.8	-	-	1.5	-	0.2	50 IM
(b)	1.3	6.0	6.0	-	-	6.8	-	3.1	
9(a)	Negl.	-	-	-	2.5	-	-	Negl.	100 IM
(b)	2.8	-	-	-	12.8	-	-	3.8	
10(a)	Negl.	0.07	0.09	-	-	0.04	-	0.04	2000 Oral
(b)	2.3	3.1	4.9	-	-	3.7	-	3.2	
11(a)	Negl.	-	-	Negl.	-	Negl.	-	Negl.	2000 Oral
(b)	3.5	-	-	5.8	-	5.0	-	4.2	
Controls (non-anaemic)									
1(a)	0.03	7.2	3.2	-	-	0.9	-	0.18	50 IV
(b)	2.8	11.0	6.5	-	-	8.3	-	4.3	
2(a)	0.01	3.3	0.6	0.1	0.02	-	0.02	0.02	50 IV
(b)	1.5	5.0	2.0	1.8	1.8	-	1.1	1.9	
3(a)	0.07	-	6.5	5.3	5.9	-	0.1	0.07	100 IM
(b)	1.3	-	8.0	8.0	6.5	-	6.7	1.8	
4(a)	0.01	0.02	0.6	0.02	-	-	0.02	Negl.	2000 Oral
(b)	0.8	2.4	8.2	3.6	-	-	1.8	2.2	
5(a)	0.05	0.06	0.06	0.07	0.02	-	0.09	0.06	2000 Oral
(b)	1.6	1.6	1.7	6.0	4.8	-	4.9	2.2	

* (a) = unheated serum, (b) = serum heated at 100°C. for 30 minutes.

the vitamin would appear to contain free B_{12} in detectable amounts, while the heated serum appears to contain free B_{12}, B_{12} that has been freed from a combined form, and the unknown growth factor that is not destroyed by alkaline hydrolosis.

It will be noted that there was only a slight rise in the serum level of B_{12} (heated samples) when large doses of the vitamin were given by mouth.

Folic Acid.

The mean resting folic acid level in the serum of 16 untreated pernicious anaemia patients was 1.21 mμg./ml. (corrected for a mean citrovorum content of 0.83 mμg./ml.). In 10 non-anaemic controls the figures were 1.5 mμg./ml. for folic acid and 0.8 mμg./ml. for citrovorum factor. The differences were not significant.

In 3 patients with megaloblastic anaemia of pregnancy refractory to cyanocobalamin therapy, the corrected folic acid levels were, respectively 1.1 mμg./ml., negligible and negligible. The citrovorum factor levels were 0.3, 0.7 and 2.0 mμg./ml. respectively. In 'sprue' with megaloblastic anaemia the corrected folic acid levels in two cases were 1.3 and 0.15 mμg./ml.: the citrovorum factor levels were 0.25 and 0.35 mμg./ml.

All that can be said as a result of this part of the investigation is that measurement of the serum content of folic acid or citrovorum factor does not help in a diagnosis of the type of megaloblastic anaemia present. It is possible to have pernicious anaemia with a negligible amount of folic acid activity in the serum and to have idiopathic steatorrhoea with severe/

severe folic acid and citrovorum factor deficiency as measured by urinary excretion tests but with relatively normal folic acid and citrovorum factor activity in the serum. It would appear that in the serum this method of assay is not, in fact, measuring only pteroylglutamic acid and citrovorum factor, but also other unknown substances the identification of which would not be practicable.

The serum levels of total folic acid activity and of citrovorum factor after the administration of folic acid are shown in Table 43. The administration of folic acid did not lead to any change in the serum level of growth factors for L.leichmannii. The main purpose of this part of the investigation was to see whether a rise of serum citrovorum factor occurred.

It will be seen that in all the cases shown in Table 43 there was a considerable rise in the serum folic acid activity following the administration of folic acid, but no appreciable rise in the serum level of citrovorum factor.

Folic Acid Conjugates.

The synthetic folic acid conjugates, pteroyldiglutamic acid and pteroyltriglutamic acid were administered to 10 untreated pernicious anaemia patients and 3 controls*, and measurements made of the various haemopoietic factors in the serum and urine. The urinary findings of these and other cases/

---

* The investigation could not be carried further since no further supplies of synthetic conjugates could be obtained.

TABLE 43.

Serum Folic Acid Activity and Level of Citrovorum Factor Following the Administration of Folic Acid.

(mμg./ml.)

Case*	0	5 min.	½ hr.	1	2	3	5	8	24hr.	FA Dose Route
Pernicious anaemia in relapse										mg.
1(a)	0.4	-	1500	610	498	-	162	-	1.4	15 IV
(b)	0.17	-	0.25	0.25	0.17	-	0.3	-	0.25	
2(a)	Negl.	-	2920	1420	-	-	-	-	-	15 IV
(b)	0.8	-	1.0	0.2	-	-	-	-	-	
3(a)	Negl.	2070	930	580	-	-	-	-	-	15 IV
(b)	1.4	1.2	1.8	1.8	-	-	-	-	-	
Megaloblastic anaemia of pregnancy**										
1(a)	Negl.	-	2000	1050	-	470	-	46	Negl.	15 IV
(b)	1.4	-	1.6	2.4	-	1.4	-	1.2	0.5	
2(a)	-	-	900	900	-	120	57	-	12.4	15 IV
(b)	-	-	4.0	4.2	-	5.8	4.6	-	4.6	
Anaemia in pregnancy (normoblastic)**										
1(a)	Negl.	-	1260	710	-	185	104	47	12	15 IV
(b)	1.0	-	0.7	1.2	-	0.8	0.8	0.7	0.7	
Megaloblastic anaemia refractory to vitamin B_{12}**										
1(a)	Negl.	-	2100	3200	-	650	-	460	-	15 IV
(b)	0.6	-	1.0	0.7	-	0.7	-	0.7	-	
Non-anaemic controls										
1(a)	3.2	-	75	430	-	400	-	190	4.4	15
(b)	6.0	-	6.0	6.2	-	7.2	-	7.2	5.4	Orally
2(a)	1.2	-	1320	600	360	-	140	-	-	15 IV
(b)	1.3	-	1.7	1.7	1.7	-	1.7	-	-	
3(a)	2.4	-	4800	2200	470	440	-	22.5	3.2	15 IV
(b)	0.2	-	2.8	1.2	1.6	1.2	-	4.0	5.0	
4(a)	2.8	-	2000	720	350	260	-	31	3.6	15 IV
(b)	1.1	-	1.2	1.1	1.3	1.2	-	1.1	0.9	

* (a) = folic acid activity (S.faecalis)
(b) = citrovorum factor content (L.citrovorum)

** I am grateful to Dr. C.C.Ungley for kindly supplying sera from these patients.

cases will be considered later. There was no measurable change in the serum content of growth factors for L.leichmannii. The results obtained with S.faecalis and L.citrovorum are given in Table 44.

Pteroyldiglutamic acid does not support the growth of the test organism for folic acid (S.faecalis) used in the assay. Under the conditions of our investigations pteroyltriglutamic acid had about one twenty-fifth the capacity of pteroylglutamic acid for supporting the growth of S.faecalis. Comparison of the results shown in Table 43 with those in Table 44 indicates that the ability of the serum to support the growth of S.faecalis following the administration of pteroyltriglutamic acid does not differ greatly from that found after the administration of pteroylglutamic acid. This, together with the urinary findings given later, would suggest that the pernicious anaemia patient is able to convert pteroyldiglutamic acid and pteroyltriglutamic acid into "free" folic acid, or perhaps into the form pteroyl-γ-glutamyl-glutamic acid, which also supports the growth of S.faecalis. This form is not known to be present in naturally occurring materials. In case 5 (Table 44) the level of folic acid activity was higher in the serum after vitamin B_{12} had been given, but such was not the case with patients 6 and 7.

It will be noted that no rise of serum citrovorum factor could be detected.

Citrovorum Factor.

The serum results will be considered together with the urinary findings.

THE/

TABLE 44.

Serum Levels of Folic Acid Activity and of Citrovorum Factor Following the Administration of Teropterin (Pteroyltriglutamic Acid) and Diopterin (Pteroyldiglutamic Acid)

(mμg./ml.)

Case*	0	5 min.	½ hr.	1	2	3	5	8	24hr.	Route	Drug & Dose
Pernicious anaemia in relapse											
1(a)	0.4	-	108	440	590	600	-	-	-	IV	Terop. 10 mg.
(b)	0.5	-	4.7	0.5	0.5	0.5	-	-	-		
2(a)	Negl.	-	720	720	340	-	130	90	Negl.	IV	Terop. 20 mg.
(b)	1.0	-	1.0	0.7	0.9	-	1.0	1.4	0.7		
3(a)	3.6	-	25	62	82	85	82	-	5.2	IV	Diop. 20 mg.
(b)	0.8	-	0.8	0.4	0.4	0.5	0.3	1.0	0.9		
4(a)	8.0	46	56	82	-	94	-	-	9.0	IV	Diop. 20 mg.
(b)	1.8	1.8	2.0	1.7	-	0.8	-	-	1.6		
5(a)	Negl.	1080	1280	1320	880	450	200	75	Negl.	IV	Terop. 20 mg.
(b)	1.01	1.18	0.9	1.01	0.68	1.01	0.85	1.18	0.85		
(a)	Negl.	3700	2100	-	-	-	-	-	-	IV	Terop. 20 mg.
(b)	0.9	0.4	0.6	-	-	-	-	-	-	IV	+Vit.B_{12} 50 μg.
6(a)	Negl.	2000	1580	1020	380	380	-	-	8.8	IV	Terop. 10 mg.
(b)	0.8	2.0	0.5	1.0	1.1	1.1	-	-	2.1		
(a)	8.8	2360	980	510	350	-	-	-	-	IV	Terop. 10 mg.
(b)	2.1	4.9	1.2	2.7	3.1	-	-	-	-	IV	+Vit.B_{12} 50 μg.
7(a)	3.6	-	1100	800	380	450	-	-	2	IV	Terop. 20 mg.
(a)	2.0	-	950	900	600	250	-	-	1.8	IV	Terop. 20 mg.
										IV	+Vit.B_{12} 50 μg.
8(a)	Negl.	-	1600	1060	-	-	-	-	Negl.	IV	Terop. 20 mg.
(b)	0.8	-	0.6	1.0	-	-	-	-	1.3		
(a)	Negl.	-	2920	1420	-	-	-	-	-	IV	FA 15 mg.
(b)	1.3	-	1.0	0.2	-	-	-	-	-		
9(a)	Negl.	340	365	360	-	-	-	-	2.4	IV	Terop. 20 mg.
(b)	1.4	1.4	1.4	1.4	-	-	-	-	1.4		
(a)	2.4	2070	930	580	-	-	-	-	2.2	IV	FA 15 mg.
(b)	1.4	1.2	1.8	1.8	-	-	-	-	1.0		
10(a)	1.6	-	-	380	2560	1060	2120	-	-	Oral	Diop. 50 mg.
(b)	1.8	-	-	1.8	1.8	1.8	2.2	-	-		
Non-anaemic controls											
1(a)	2.0	780	1270	720	740	685	240	-	2.0	IV	Terop. 20 mg.
(b)	0.8	1.7	1.5	1.6	3.0	1.6	1.4	-	0.8		
2(a)	2.8	3200	-	720	150	220	-	33	2.0	IV	Terop. 20 mg.
(b)	2.4	3.8	-	5.3	1.4	0.4	-	Negl.	0.4		
3(a)	1.2	120	96	110	-	72	-	-	-	IV	Diop. 15 mg.
(b)	1.2	2.8	1.4	1.6	-	1.7	-	-	-		

* (a) = folic acid activity (S.faecalis)
(b) = citrovorum factor content (L.citrovorum)

THE ESTIMATION OF HAEMOPOIETIC SUBSTANCES IN THE URINE

Vitamin B_{12}.

We have found that the content of vitamin B_{12} as measured by the L.leichmannii assay is of a similar order of magnitude in the urines of pernicious anaemia patients and controls. In the urines of 30 untreated pernicious anaemia patients the mean excretion in 24 hours was 97.2 mμg. (This is the mean of 25 observations as in 5 patients the value was too low to be measured). The range was nil-350 mμg./24 hours. In 30 non-anaemic controls the mean of 26 observations was 117.9 mμg./24 hours with a range of nil-500 mμg./24 hours. The difference is not significant ($t = .73$, $P = .5$). Hoffman et al. (1949) stated that vitamin B_{12} was destroyed by being heated with 0.2 N NaOH at 100°C. for 30 minutes and that under these conditions the desoxyribosides of thymine, guanine and hypoxanthine (which can replace vitamin B_{12} in the L.leichmannii assay) were not affected. In the present investigation it was found that alkaline treatment of this nature would destroy most of the growth factors for L.leichmannii present in the urine, and that it would destroy vitamin B_{12} that had been added to the urine. The figures given above are for growth factors for L.leichmannii that could be destroyed in this way. On the other hand, Robinson et al. (1952) considered that vitamin B_{12} could be destroyed in solution if boiled for 30 minutes at pH 10.

In the course of the present investigation, 20 μg. of cyanocobalamin were added to a litre of urine of a pernicious anaemia patient. The urine was then assayed for vitamin B_{12} and/

and it was found that the added amount could be recovered without loss, and that it could be destroyed by alkaline hydrolysis performed by the method of Hoffman et al. (1949). When, however, the urine was boiled for 30 minutes at pH 11, 14.2 μg. of vitamin B_{12} could be recovered. Similar results were obtained with the urine of a normal person. (The reports of Robinson et al. (1952) refer to cup plate assays performed on liver extracts, and not to urinary assays).

A number of samples of urine were then boiled at 100°C. for 30 minutes both at pH 6.8 and at pH 11.0. As would be expected, in most instances heating at pH 11 reduced the ability of the urine to support the growth of L.leichmannii, but in some of the non-anaemic patients, the growth-promoting activity of the urine for L.leichmannii was increased (even as much as seven-fold in one instance) by this treatment. Such effects were, however, erratic and difficult to reproduce: they were not found in the urines of pernicious anaemia patients. It seemed likely that growth factors for L.leichmannii were being released from some "combined" form. Further alkalinisation destroyed this activity. These results are shown in Tables 45 and 46.

It was considered that L.leichmannii was not a satisfactory organism for the estimation of the vitamin B_{12} content of the urines of patients who were not receiving cyanocobalamin.therapy.

When cyanocobalamin had been administered, our results on the urinary excretion of this factor were similar to those of other workers (Chesterman et al. (1951); Conley et al.(1951)) and/

TABLE 45

EFFECT ON APPARENT VITAMIN B_{12} CONTENT OF URINES (L.leichmannii assay) OF HEATING AT pH 11 FOR 30 MINUTES

Case Diagnosis	Vitamin B_{12} content mμg./24 hrs. Untreated urine	Heated 30 mins. at pH 11 (100° C.)	Heated 30 mins. with 0.2N NaOH (100° C.)
Normal	1. 142	502	121
	2. 71	80	50
Duodenal ulcer	43	294	43
Coronary thrombosis	81	275	52
Pregnancy	182	341	Negligible
Hypertension	90	150	30
Jejunal cancer	37	70	37
Peptic ulcer	162	226	24
Iron deficiency	429	533	0
Peptic ulcer	443	150	Negligible
Iron deficiency	99	91	37
Unresolved pneumonia	88	41	27
Normal	48	44	14

TABLE 46

EFFECT ON APPARENT VITAMIN B_{12} CONTENT OF URINES (L.leichmannii assay) OF HEATING AT pH 11 FOR 30 MINUTES

(Megaloblastic anaemia patients)

	Vitamin B_{12} content mμg./24 hrs.		
	Untreated urine	Heated 30 mins. at pH 11 (100°C.)	Heated 30 mins. with 0.2N NaOH (100°C.)
Pernicious anaemia			
C	420	248	41
R	214	245	negligible
D	199	92	31
B	122 * 20	negligible 396	negligible 20
T	118	148	58
Te	16	negligible	negligible
Y	9	negligible	negligible
Megablastic anaemia of pregnancy			
S	224	90	negligible
D	74	218	53
Sprue			
W	Negligible	negligible	negligible

* After having been kept unfrozen in refrigerator for a few weeks.

and hence need not be reported in detail. When doses of 40 or 50 μg. were given parenterally to 12 subjects (8 with untreated pernicious anaemia) the urinary excretion did not exceed 7.4 μg. in any, whereas when 100 μg. was given to 7 subjects (5 with untreated pernicious anaemia) the amount excreted ranged from 16.6 μg. to 38.5 μg. When 2,000 μg. were given by mouth to 7 subjects (4 with untreated pernicious anaemia) there was no measurable excretion, although there was a haematological response in the pernicious anaemia patients. Assays with B.coli as test organism gave similar results.

There was nothing in these few results to suggest that the output following the parenteral or oral administration of cyanocobalamin differed in pernicious anaemia patients from controls.

Heating the urine at pH 11 did not increase the urinary content of growth factors for L.leichmannii.

The administration of cyanocobalamin did not alter measurably the urinary content of folic acid or citrovorum factor in the 24 hours following its administration. Some results of estimations of urinary folic acid activity for several days after an injection of cyanocobalamin are given in Fig.7.

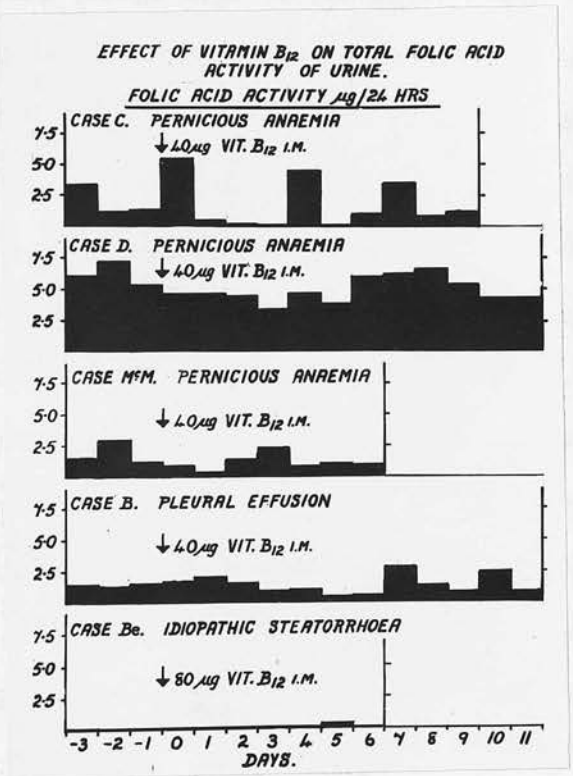

EFFECT OF VITAMIN B12 ON TOTAL FOLIC ACID ACTIVITY OF URINE.
FOLIC ACID ACTIVITY µg/24 HRS
CASE C. PERNICIOUS ANAEMIA
↓40µg VIT. B12 I.M.
CASE D. PERNICIOUS ANAEMIA
↓40µg VIT. B12 I.M.
CASE McM. PERNICIOUS ANAEMIA
↓40µg VIT. B12 I.M.
CASE B. PLEURAL EFFUSION
↓40µg VIT. B12 I.M.
CASE Be. IDIOPATHIC STEATORRHOEA
↓80µg VIT. B12 I.M.
7·5
5·0
2·5
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11
DAYS.

Fig. 7.

Folic Acid.

The mean folic acid content of the urine of 17 untreated pernicious anaemia patients (corrected for citrovorum factor) was 1.8 μg./24 hours (range 0.05 to 3.7 μg.). The mean citrovorum content was 0.79 μg./24 hours (range 0.1 to 2.9 μg.). In 10 non-anaemic controls the mean folic acid content was 1.7 μg./24 hours (range 0.12 to 3.6 μg.). The mean citrovorum content was 0.84 μg./24 hours (range negligible (one case) to 3.3 μg.). The differences were not significant.

At an early stage in our investigations we found, in confirmation of the work of Bethell et al. (1947) that pernicious anaemia patients excrete in the urine less of a test dose of pteroylglutamic acid than do controls. Consideration of the absorption of folic acid from the small intestine will be deferred until Chapter 9. In their investigations Bethell and his co-workers gave the folic acid by mouth, but we preferred to inject it subcutaneously. A summary of the findings in 20 untreated pernicious anaemia patients and 20 non-anaemic controls is given in Fig.8.

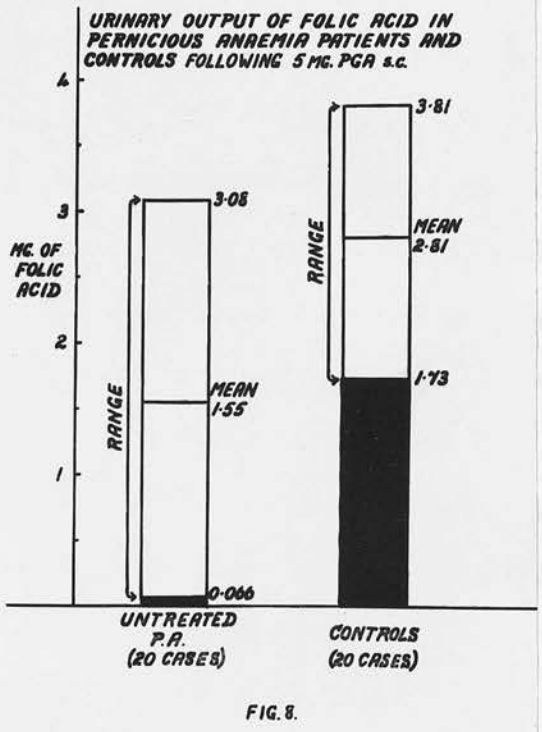

URINARY OUTPUT OF FOLIC ACID IN PERNICIOUS ANAEMIA PATIENTS AND CONTROLS FOLLOWING 5 MG. PGA s.c.
MG. OF FOLIC ACID
4
3
2
1
3·08
MEAN 1·55
0·066
RANGE
3·81
MEAN 2·81
1·73
RANGE
UNTREATED P.A. (20 CASES)
CONTROLS (20 CASES)

FIG. 8.

It is not, however, entirely satisfactory to use non-anaemic controls and our experience with folic acid excretion tests in a wide variety of conditions is the subject of a separate section of this thesis. The results may be anticipated here to the extent of the making of a statement that following a 5 mg. test dose of pteroylglutamic acid the mean urinary folic acid output in a series of untreated pernicious anaemia patients is less than in a series of anaemic or non-anaemic controls. Other forms of megaloblastic anaemia, malignant disease, renal failure or a large effusion may also be associated with the excretion of less folic acid after the test dose. The presence or absence of a diminished output of folic acid in the urine in untreated pernicious anaemia does not necessarily bear a relationship to the level of the erythrocyte count.

It has been shown by Broquist et al. (1951) and by Spray & Witts (1951, 1952) that normal persons given folic acid by mouth can excrete approximately 0.1 per cent. of this in the urine as citrovorum factor. This is similar to our findings which need not therefore be detailed.

There was no measurable change in the urinary content of vitamin B_{12} (L.leichmannii assay) following the administration of folic acid.

Folic Acid Conjugates.

Numerous workers have shown that it is possible to treat pernicious anaemia patients successfully not only with pteroylglutamic acid itself, but also with pteroyldiglutamic acid and pteroyltriglutamic acid. This was our experience (see p. 403).

Under/

Under the conditions of our assay, 10 mg. of pteroyltriglutamic acid will support the growth of S.faecalis to the same extent as 0.38 mg. of pteroylglutamic acid. Pteroyldiglutamic acid does not support the growth of S.faecalis. It has been reported by Jukes & Stokstad (1948) that such conjugates can be converted in the normal person to folic acid, and excreted as such in the urine.

In Table 47 there are given the results of estimations of the urinary folic acid activity and citrovorum factor content following the administration of pteroyldiglutamic acid and pteroyltriglutamic acid to pernicious anaemia patients and controls. No attempts were made in the present investigation to carry out a detailed comparison of the urinary output of folic acid in pernicious anaemia patients and normal persons under these conditions, partly because we were unable to obtain further supplies of folic acid conjugates after the completion of the earlier stages of this investigation. Such a study would probably require the use of smaller quantities of folic acid conjugates than the 20 mg. given to the three control cases in Table 47.

It will be seen that the urinary findings, like those of the serum, suggest that the pernicious anaemia patient is able to convert the synthetic folic acid conjugates into folic acid (or perhaps into some other substance with folic acid activity, possibly pteroyl-γ-glutamyl-glutamic acid), since the urinary excretion following the administration of pteroyltriglutamic acid is frequently greater than can be explained by the activity of this substance itself to support the growth of S.faecalis./

TABLE 47

URINARY FOLIC ACID ACTIVITY AND CITROVORUM FACTOR CONTENT FOLLOWING THE ADMINISTRATION OF SYNTHETIC FOLIC ACID CONJUGATES

Case	Drug used	Dose mg.	Route	Urinary Folic acid activity mg. in 24 hrs. after treatment	Urinary citrovorum factor µg. in 24 hrs. after treatment
		Pernicious anaemia (in relapse)			
1	Pteroyl-triglutamic acid	20	Iv.	5.99	11.4
2		20	Iv.	1.94	2.1
3		10	Iv.	0.15	4.2
		10 + 50 µg.B_{12}	Iv.	0.15	1.8
4		20	Iv.	7.10	8.3
		20 + 50 µg.B_{12}	Iv.	5.50	7.7
5		10	Im.	1.88	-
6		10	Oral	1.44	-
7		2.5	Oral	0.036	-
8		2.5	Oral	0.006	0.48
9	Pteroyl-diglutamic acid	50	Oral	10.09	16.2
10		20	Iv.	1.104	3.9
11		20	Iv.	0.396	3.6
12		5	Im.	0.28	2.8
13		5	Oral	0.27	2.2
14		5	Oral	0.33	0.2
15		2.5	Im.	0.012	0.98
16		2.5	Im.	0.37	0.09
		Non-anaemic persons			
1	Pteroyl-triglutamic acid	20	Iv.	3.47	4.9
2		20	Iv.	9.20	10.5
3	Pteroyl-diglutamic acid	15	Iv.	1.31	3.2

S.faecalis.

The rise of urinary content of citrovorum factor following the administration of the synthetic conjugates was slight. We did not obtain sufficient data to enable a comparison to be made with the output of citrovorum factor following the administration of folic acid to pernicious anaemia patients.

There was no change in the urinary content of growth factors for L.leichmannii.

Citrovorum Factor.

The synthetic preparation of citrovorum factor Leucovorin was given to 2 normal persons by mouth and to one by injection. It was also administered by intramuscular injection to 2 untreated pernicious anaemia patients, and by mouth to a patient with pernicious anaemia in relapse and to a man with histamine fast achlorhydria whodid not suffer from pernicious anaemia. Serum and urinary levels of citrovorum factor activity were then measured using both L.citrovorum and S.faecalis as the test organism.

Since the growth of L.citrovorum under these conditions depends on the presence of citrovorum factor but not of folic acid in the serum or urine, whereas the growth of S.faecalis is stimulated either by citrovorum factor or folic acid, the growth of S.faecalis to an extent greater than that of L.citrovorum must be taken to indicate the presence of folic acid (or some unknown related substance) in the serum or urine undergoing test. Any conversion of citrovorum factor to folic acid (or related substance) in the body should therefore be apparent from the double assay.

The/

The results of these investigations are shown in Figures 9 to 15. There was no change in the serum content of growth factors for L.leichmannii.

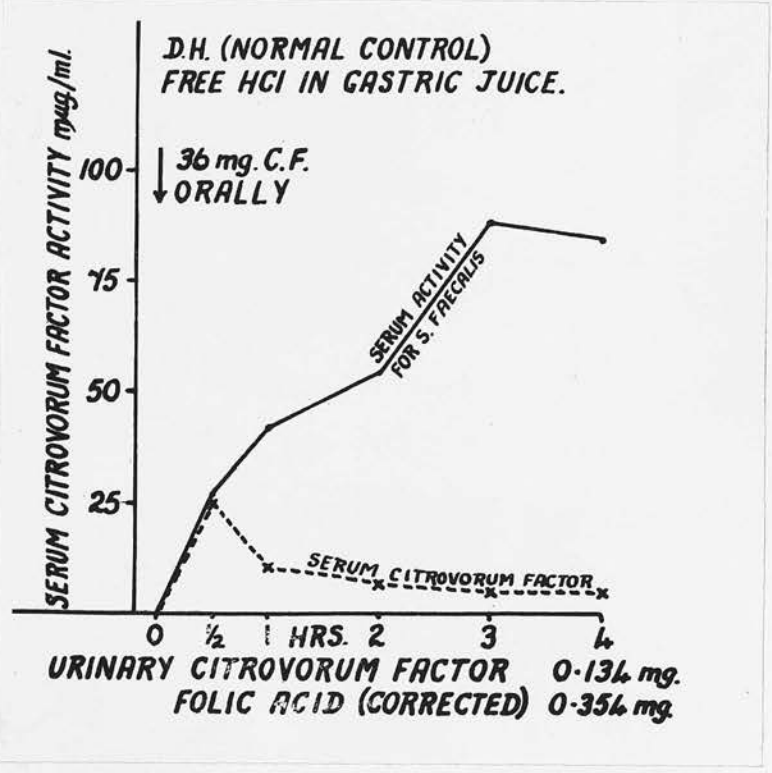


Fig. 9.

Figs. 9-15: The metabolism of citrovorum factor in pernicious anaemia patients in relapse and in others.

Note: CF. indicates the administration of the synthetic preparation of citrovorum factor Leucovorin.

In these graphs, serum activity for S.faecalis is measured with a citrovorum factor standard. This activity is due to citrovorum factor itself, plus any "folic acid" that may be present. The serum citrovorum factor is measured with the same standard, but the organism Leuconostoc citrovorum which is used for the assay measures only citrovorum factor itself and no folic acid. The urinary findings are for a 24 hour output, and the urinary folic acid has been corrected for activity due to citrovorum factor.

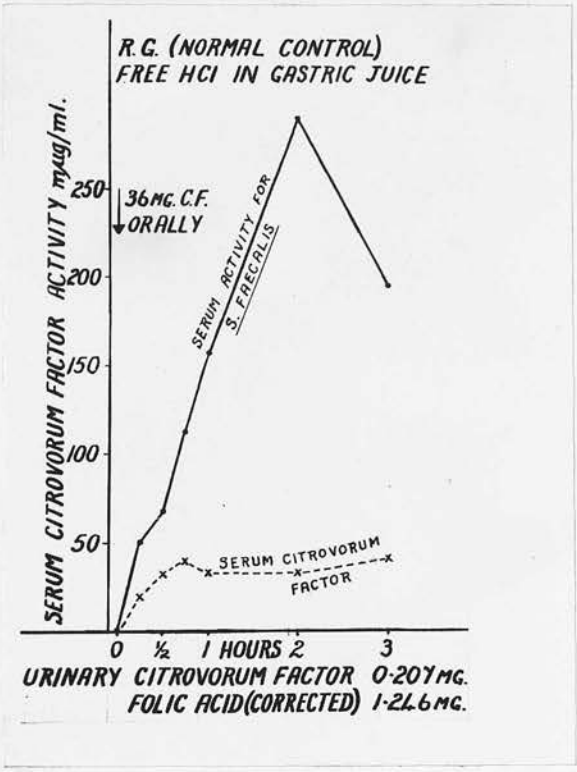

R.G. (NORMAL CONTROL)
FREE HCl IN GASTRIC JUICE
SERUM CITROVORUM FACTOR ACTIVITY mμg/ml.
250
200
150
100
50
36 MG. C.F.
ORALLY
SERUM ACTIVITY FOR S. FAECALIS
SERUM CITROVORUM FACTOR
0 ½ 1 HOURS 2 3
URINARY CITROVORUM FACTOR 0·207 MG.
FOLIC ACID (CORRECTED) 1·24·6 MG.

Fig. 10.

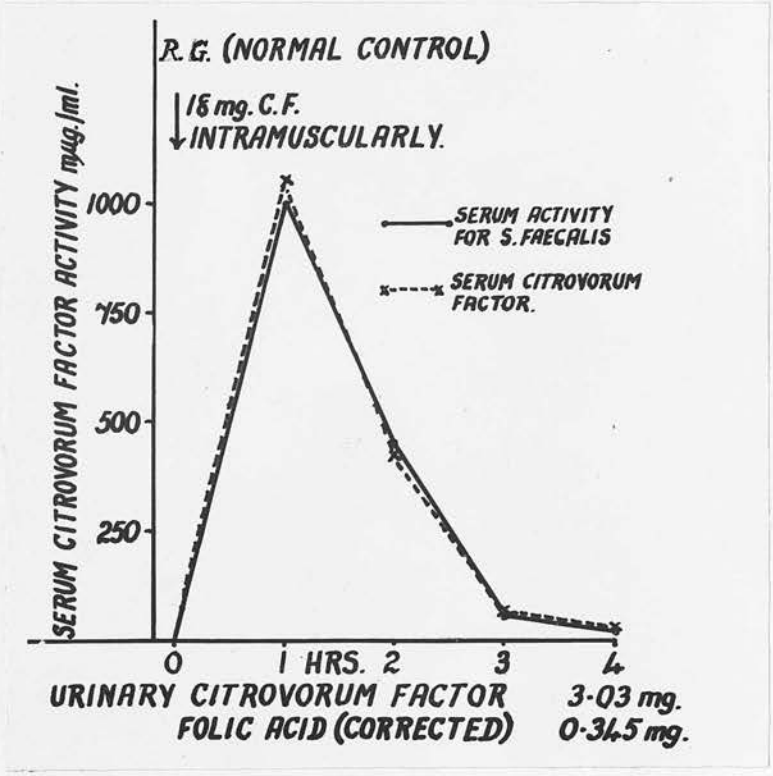

R.G. (NORMAL CONTROL)
18 mg. C.F.
INTRAMUSCULARLY.
SERUM CITROVORUM FACTOR ACTIVITY mμg./ml.
1000
750
500
250
SERUM ACTIVITY FOR S. FAECALIS
SERUM CITROVORUM FACTOR.
0 1 HRS. 2 3 4
URINARY CITROVORUM FACTOR 3·03 mg.
FOLIC ACID (CORRECTED) 0·34·5 mg.

Fig. 11.

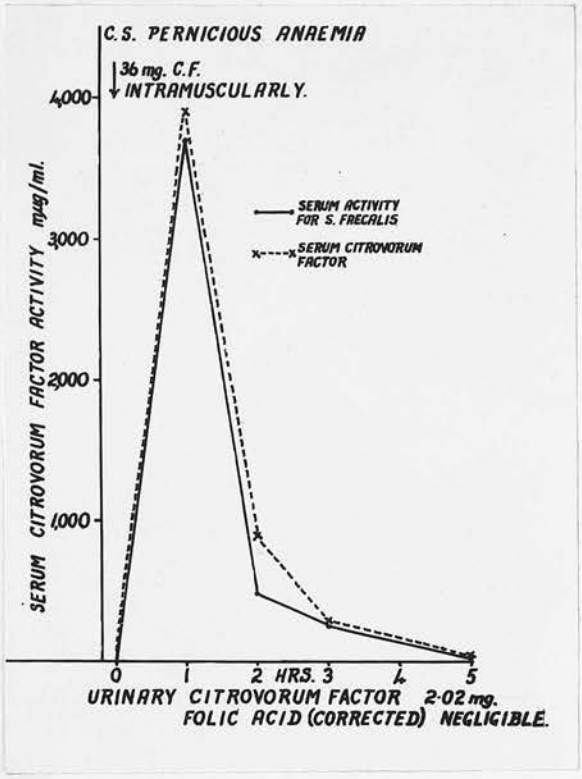

C.S. PERNICIOUS ANAEMIA
36 mg. C.F.
INTRAMUSCULARLY.
SERUM CITROVORUM FACTOR ACTIVITY mμg/ml.
4,000
3,000
2,000
1,000
SERUM ACTIVITY FOR S. FAECALIS
SERUM CITROVORUM FACTOR
0 1 2 HRS. 3 4 5
URINARY CITROVORUM FACTOR 2·02 mg.
FOLIC ACID (CORRECTED) NEGLIGIBLE.

Fig. 12.

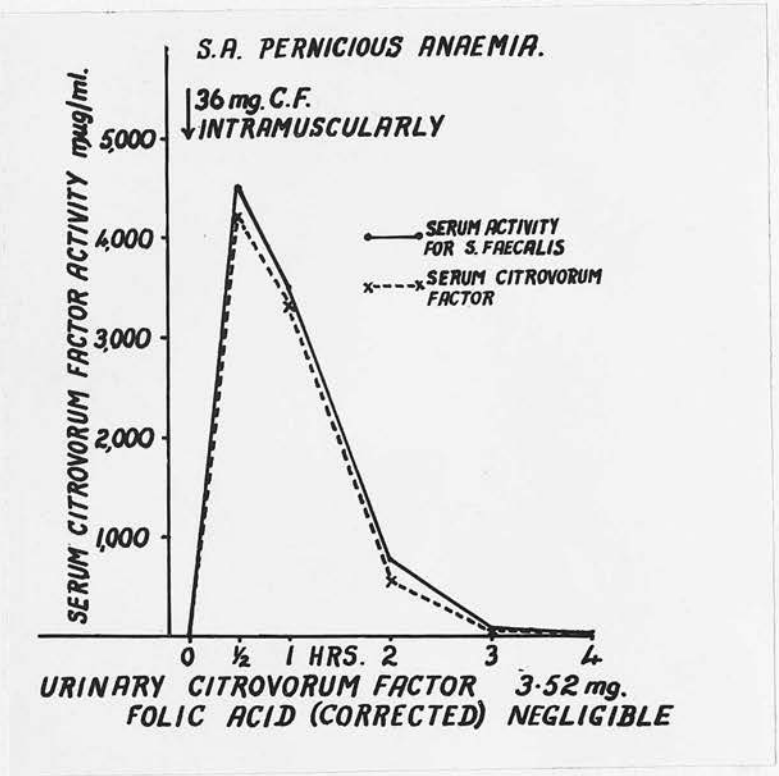

S.A. PERNICIOUS ANAEMIA.
36 mg. C.F.
INTRAMUSCULARLY
SERUM CITROVORUM FACTOR ACTIVITY mμg/ml.
5,000
4,000
3,000
2,000
1,000
SERUM ACTIVITY FOR S. FAECALIS
SERUM CITROVORUM FACTOR
0 ½ 1 HRS. 2 3 4
URINARY CITROVORUM FACTOR 3·52 mg.
FOLIC ACID (CORRECTED) NEGLIGIBLE

Fig. 13.

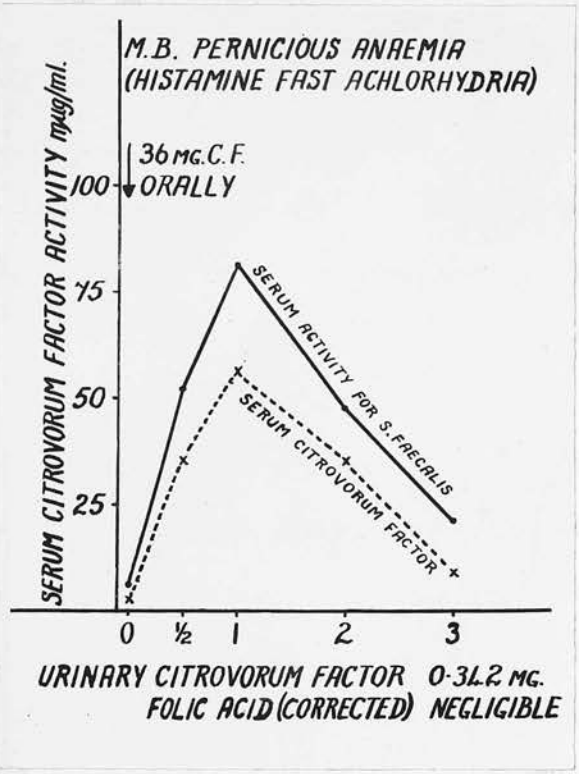

M.B. PERNICIOUS ANAEMIA
(HISTAMINE FAST ACHLORHYDRIA)
36 MG. C.F.
ORALLY
SERUM CITROVORUM FACTOR ACTIVITY mμg/ml.
100
75
50
25
SERUM ACTIVITY FOR S. FAECALIS
SERUM CITROVORUM FACTOR
0
½
1
2
3
URINARY CITROVORUM FACTOR 0·34·2 MG.
FOLIC ACID (CORRECTED) NEGLIGIBLE

Fig. 14.

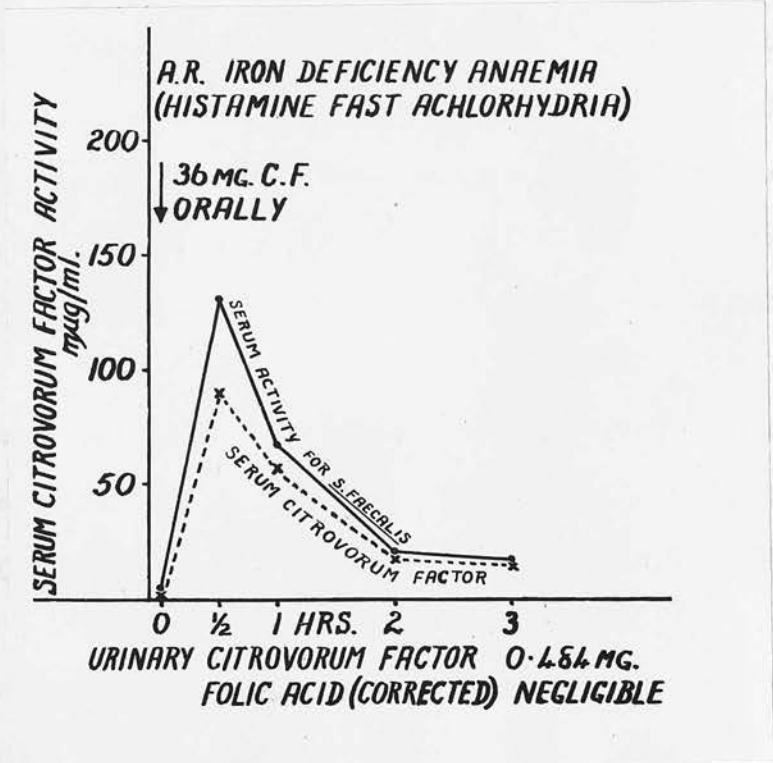

A.R. IRON DEFICIENCY ANAEMIA
(HISTAMINE FAST ACHLORHYDRIA)
36 MG. C.F.
ORALLY
SERUM CITROVORUM FACTOR ACTIVITY mμg/ml.
200
150
100
50
SERUM ACTIVITY FOR S. FAECALIS
SERUM CITROVORUM FACTOR
0
½
1 HRS.
2
3
URINARY CITROVORUM FACTOR 0·4·84 MG.
FOLIC ACID (CORRECTED) NEGLIGIBLE

Fig. 15.

The results summarised in Figures 9 to 15 would appear to indicate that in a normal person orally administered citrovorum factor may appear in the serum and in the urine largely as folic acid. (As has been indicated above, this may, in fact, be a related substance, but for convenience the term folic acid will be used). The two normal control patients had free hydrochloric acid in the gastric juice. In the two patients with histamine fast achlorhydria, one of whom had pernicious anaemia, the citrovorum factor appeared in the serum and urine largely as citrovorum factor. This was also true of the patients and control who were given the citrovorum factor intramuscularly.

The most likely explanation of these findings would appear to be that citrovorum factor is normally converted by the acid of the gastric juice into folic acid and absorbed as such. To test this hypothesis further, 1 mg. quantities of citrovorum factor were incubated at 37°C. for 2 hours with 40 ml. each of (a) normal gastric juice at pH 2.8, (b) pernicious anaemia gastric juice at its pH of 7.8, and (c) pernicious anaemia gastric juice adjusted to pH 2.8 with hydrochloric acid.

Some conversion to folic acid occurred in all three.

The ratios growth factors for S.faecalis/growth factors for L.citrovorum were 2.3:1, 1.5:1, and 2.7:1 in samples (a), (b), and (c) respectively after incubation; citrovorum factor is readily converted by acid to folic acid, and hence it is reasonable to assume that such conversion occurs in the stomach of a patient with free hydrochloric acid.

A/

A few further observations on citrovorum factor excretion after injected and orally administered test doses will be found in Chapter 12.

DISCUSSION.

The problems of the metabolic interrelationships of the antimegaloblastic haemopoietic substances are complex and as yet little understood; the possible growth factors required by the various organisms used in testing for these substances also require further elucidation.

It would appear likely that heating of the serum of pernicious anaemia patients or controls makes available for the growth of L.leichmannii some substance or substances other than vitamin B_{12} itself. In the light of present knowledge it would seem likely that desoxyribosides of substances such as thymine, guanine or hypoxanthine are made available by such heating. Ross (1950) has suggested that when vitamin B12 has been administered to patients by injection it is found first in a "free" form and later partly in a "combined" form. Heating the serum makes "combined" vitamin B_{12} available for the growth of Euglena gracilis.

This would also appear to be true with L.leichmannii which, however, is not a suitable organism for making such measurements by the techniques outlined here.

Our recovery experiments, where vitamin B_{12} had been added to the serum (see p. 274) suggest that the vitamin may enter into some form of combination in the serum in vitro as well as in vivo. Further experiments with B.coli as the test organism showed that the growth factors for L.leichmannii which

are/

are released by heating the serum of untreated pernicious anaemia patients and controls do not support the growth of B.coli. However this organism is too insensitive for employment in assaying the serum for its content of vitamin B_{12}. In the chapter that follows an account is given of the measurement of serum vitamin B_{12} levels where the serum proteins were precipitated before the assay was carried out. When this was done it was found possible to demonstrate a difference between untreated pernicious anaemia patients and controls in this respect without the complication of unknown growth factors for L.leichmannii being present.

While recovery experiments performed on urines with added vitamin B_{12} have been satisfactory where L.leichmannii was the test organism employed, it will be seen that with it no significant difference could be found between the B_{12} content of the urines of pernicious anaemia patients and controls. This is in contrast with the findings of Mollin & Ross (1952) who used Euglena gracilis for the test. This may indicate that the L.leichmannii assay measures unknown substances other than vitamin B_{12} which are destroyed by alkaline hydrolysis, but the surprising feature is that our results on the normal controls are in fairly good agreement with the findings of Mollin & Ross (a mean of 0.12 µg./24 hours urinary output compared with their figure of 0.18 µg./24 hours). In contrast, our mean figure for the urines of pernicious anaemia patients was 0.09 µg./24 hours, whereas their mean figure was less than 0.05 µg./24 hours. At present it would seem that the/

the Euglena assay is the method of choice for urinary assays.

It is also surprising but a matter upon which the various investigators are in agreement, that the urinary excretion of parenterally administered vitamin B_{12} is of the same order of magnitude in pernicious anaemia patients as it is in controls. This will be discussed further in Chapter 12. The practical point is that it is not possible to devise a urinary vitamin B_{12} excretion test that will assist in the diagnosis of pernicious anaemia.

Our findings indicate, too, that it is not feasible to diagnose the type of megaloblastic anaemia present by measuring the serum content of folic acid or citrovorum factor.

As the urinary excretion of citrovorum factor following the administration of folic acid is small, it is perhaps not surprising that there is no measurable rise in the serum content of citrovorum factor even when the serum folic acid level is very high.

As Bethell et al. (1947) and others have shown, we have found that the urinary output of folic acid following a test dose of pteroylglutamic acid is less in a group of untreated pernicious anaemia patients than in non-anaemic controls.

Our estimations of the serum folic acid content following the administration of the synthetic conjugates of folic acid would appear to indicate that the pernicious anaemia patient is able to convert these conjugates rapidly into folic acid itself, or into some intermediate compound with folic acid activity. When 2 mg. of pteroyltriglutamic acid were incubated with

100 ml./

100 ml. of plasma for 2 hours, only very slight conversion of this nature occurred. Unfortunately, however, these conjugates are not the forms that occur naturally and hence these investigations do not help us to understand the fault in metabolism of folic acid conjugates (and possibly citrovorum factor conjugates) that may occur in pernicious anaemia. It will be recalled that Bethell et al. (1947) and others have suggested that in pernicious anaemia there is a defect in the conversion of conjugated folic acid to the free form.

The experiments with orally administered citrovorum factor may be interpreted as indicating that citrovorum factor present in the diet is largely converted by the hydrochloric acid in the stomach into folic acid, but that where there is no free hydrochloric acid, absorption as citrovorum factor may occur.

SUMMARY.

1. When the sera of pernicious anaemia patients or controls were heated at 100°C. for 30 minutes, they developed the ability to support the growth of L.leichmannii by virtue of some substance other than vitamin B_{12}. In addition, however, when cyanocobalamin had been administered, such heating liberated "free" vitamin B_{12} in the serum from a combined form.

2. The L.leichmannii method of assay as performed in these investigations, unlike the method described in the following chapter, was not satisfactory for showing possible differences in the levels of vitamin B_{12} in the sera of pernicious anaemia patients and controls.

3. Serum folic acid or citrovorum factor tests did not assist in/

in the diagnosis of the type of megaloblastic anaemia present probably because they were not measuring only pteroylglutamic acid and citrovorum factor.

4. The L.leichmannii assay was of no value in showing possible differences in the urinary concentration of vitamin B_{12} in pernicious anaemia patients and controls.

5. Parenterally administered vitamin B_{12} did not cause any measurable rise in the serum level of folic acid or citrovorum factor in pernicious anaemia patients or controls.

6. The synthetic folic acid conjugates pteroyldiglutamic acid and pteroyltriglutamic acid appeared in the sera and urines of pernicious anaemia patients as pteroylglutamic acid or some related substance with folic acid activity for S.faecalis.

7. Orally administered citrovorum factor appeared to be largely converted by the gastric juice to folic acid if free hydrochloric acid was present. When administered parenterally, however, citrovorum factor was excreted in the urine largely unchanged.

CHAPTER 8.

A NEW METHOD FOR THE ESTIMATION OF SERUM LEVELS OF VITAMIN B_{12} USING L.LEICHMANNII AS THE TEST ORGANISM.

In Chapter 7 we have shown that a straightforward assay of diluted serum for its content of vitamin B_{12} with L.leichmannii as the test organism is of no value because of the presence of other growth factors for this lactobacillus. As yet the only method of assay that has been reported to show a difference in serum levels in pernicious anaemia patients and controls is that involving the use of the protozoon Euglena gracilis, and it suffers from the defect that growth has to continue for seven days before a result is available.

In November 1952, however, Rosenthal & Sarett in New Orleans stated that they could measure the vitamin B_{12} activity of serum using L.leichmannii. The method employed differed from ours in that the serum was treated with acetate buffer before the assay was performed, the medium differed in certain ways, incubation was for 65 to 72 hours in contrast to our 18 hours, and the measurement after incubation was by titration with 0.1 N alkali instead of by measurement of turbidity of organisms. We have found, however, that satisfactory results can be obtained if we treat the serum with acetate and modify our medium but retain our turbidity method of measurement of results and leave the samples in the incubator for the standard

18/

18 hours.

The modification of the medium consists particularly of cutting down the concentration of acetate. Otherwise excess acetate ions are present because the serum is treated with acetate and there is already a high concentration in our standard medium. This excess interferes with growth, and hence we used medium 3 as described on p.171.

At the time of writing, the paper by Rosenthal & Sarett does not appear to have attracted attention in Britain or amongst haematologists elsewhere. We have, however, been in communication with these workers at New Orleans and have acquainted them of our preliminary results, and have been informed that they, too, are pursuing this matter, using their own rather different technique.

METHODS

Blood is obtained by venipuncture and allowed to clot thoroughly at room temperature. The serum is removed after centrifugation and stored at -20° C.

To 5 ml. of serum there are added 5 ml. of 1 per cent acetate buffer at pH 4.6 and 20 ml. of distilled water. This provides a 1:6 dilution of serum at pH 5.1. After thorough mixing the serum bottle is plugged with cotton wool and immersed in a boiling water bath at 100° C. for 30 minutes. After cooling, the serum is centrifuged, and 22 or 23 ml. of clear supernatent is obtained. 9 ml. of this solution is adjusted to pH 6.9 with dilute sodium hydroxide and made up to a total volume of 15 ml. At this point the dilution is 1:10.

The/

The material is assayed for total vitamin B_{12} activity at levels of 1, 2,3,4 and 5 ml. per tube. Determinations are made at least in duplicate.

One of the difficulties that we have encountered is that to carry out the estimation satisfactorily it is necessary to have 5 ml. of serum for each test and therefore 10 ml. are required for the duplicate assay. For this reason repeated assays cannot normally be carried out. Microbiological methods of assay frequently involve the research worker in great technical difficulties. For example, growth of the test organism may occur in the absence of vitamin B_{12} because some vitamin B_{12} is present in one constituent of the medium (almost certainly the hydrolysed casein), or satisfactory growth may not occur at all. The latter occurrence is usually because of deterioration of the biotin in the medium. In the course of our investigations into serum levels of vitamin B_{12} we had a great deal of trouble from a combination of a high blank and a poor top level of growth; indeed for nearly two months no satisfactory results were obtained. During this period each of the ingredients of the medium was replaced, new standard cyanocobalamin was used, and a new strain of L.leichmannii was obtained by the kindness of Dr. W. F. J. Cuthbertson of Glaxo Laboratories, Ltd. It was finally noticed that two batches of hydrolysed casein supplied by the same manufacturer differed in their smell. Neither batch gave satisfactory results when used in the medium, but if the two batches were mixed together, growth proceeded,/

proceeded, albeit with a high blank. It would appear that each of the two batches was deficient in some constituent, but that the missing substance differed in the two.

This is being stressed here, because if growth fails in all the tubes, or if excess growth occurs in them all, the only damage done is that the sample of blood is wasted. On the other hand, if an inexperienced worker attempted to carry out such an assay and did not have sufficient previous knowledge to know the shape of curve that should be produced by growth of the test organism in his standard tubes, he might be in the unfortunate position of having the correct amount of growth in the tubes containing serum but not in the tubes of cyanocobalamin standard. This would lead him to report a much higher serum vitamin B12 level than was actually present. Such an occurrence may theoretically be found if the medium is deficient in some trace substance that is present in serum. In fact, however, our results of triplicate assays were as shown in Table 48. Recovery experiments with 0.1 mμg. of added cyanocobalamin per ml. of serum gave 90% recovery.

TABLE/

TABLE 48.

Results of Triplicate Assays of the Serum Level of Vitamin B_{12} in Ten Patients.

Case	Serum vitamin B_{12} ($\mu\mu$g./ml.)		(*L.leichmannii* assay)
1	< 50	< 50	< 50
2	< 50	< 50	< 50
3	< 50	< 50	80
4	50	80	60
5	100	140	130
6	150	160	160
7	180	280	230
8	185	185	200
9	200	270	240
10	430	450	300

With the kind cooperation of Dr. G.I.M.Ross of the Post-graduate School of London, specimens of serum were exchanged and estimated in Edinburgh by the *L.leichmannii* technique and in London by the *Euglena* method. The diagnosis was unknown to the recipient in each instance, and the results of the assays were posted on the same morning. The assays were done in duplicate, except that, because of technical difficulties on one day of the investigations, three of the specimens received from London were assayed only once by the *L.leichmannii* technique. The results are given in Table 49.

TABLE/

TABLE 49.

Duplicate Assays of Serum for its Vitamin B_{12} Content by two Techniques; Specimens exchanged between London and Edinburgh.

	Serum vitamin B_{12} levels ($\mu\mu$g/ml.)	L.leichmannii	Euglena gracilis
	Serum sent from London.		
	Diagnosis		
1.	Pernicious anaemia	80	60
2.	Pernicious anaemia	98	50
3.	Pooled sera	310	365
4.	Pooled sera	370	340
5.	Pooled sera with added Vit.B_{12}	490	520
6.	Atypical leukaemia	870	1250
	Serum sent from Edinburgh.		
	Diagnosis		
7.	Subacute combined: normoblastic marrow	50	50
8.	Pernicious anaemia in relapse	125	50
9.	Pernicious anaemia in relapse	120	215
10.	Pernicious anaemia in relapse	150	180
11.	Pernicious anaemia - treated	300	185
12.	Iron deficiency anaemia	320	195
13.	Neurosis	410	380
14.	Amoebiasis	440	540

The comparative results shown in Table 49 were considered to be very satisfactory, particularly since such a small quantity of a substance is being estimated. It will be noted that there was one discrepancy in that a patient with all the criteria for a diagnosis of pernicious anaemia, who subsequently responded/

responded to cyanocobalamin therapy, had a low serum vitamin B_{12} reading by the L.leichmannii method, but not by the Euglena method. The Euglena assay infrequently shows a high reading in cases of pernicious anaemia and, as will be seen in Table 50 the same is occasionally true of the L.leichmannii assay.

The results of serum vitamin B_{12} assays in 103 patients are given in Tables 50a, b, c, d. The table includes the results in 22 cases of pernicious anaemia in relapse, 9 additional patients with pernicious anaemia under treatment, 24 patients with other forms of megaloblastic anaemia or of idiopathic steatorrhoea without megaloblastic changed, and 48 other persons.

TABLE 50a.

Serum Vitamin B_{12} Levels (L.leichmannii assay) in Patients with Untreated Pernicious Anaemia.

Case	Sex	Age	Hb. G/100ml.	RBCs Mills/c.mm.	Serum Vit.B_{12} level µµg./ml.
1	F	69	6.1	1.5	< 50
2	F	59	6.8	1.9	< 50
3	M	56	7.0	2.2	< 50
4	F	52	7.6	2.3	< 50
5	M	64	10.4	2.4	< 50
6	F	57	10.5	2.5	< 50
7	F	56	10.4	2.6	< 50
8	F	77	5.2	1.4	55
9	F	29	12.1	3.8	60
10	M	53	4.4	1.3	70
11	M	70	5.8	1.6	70
12	F	64	5.9	1.7	75
13	F	60	7.2	1.7	80
14	F	68	7.4	2.2	85
15	F	26	6.1	1.7	90
16	F	60	6.7	2.0	95
17	F	69	6.2	1.1	100
18	M	53	6.2	1.7	120
19	F	43	7.7	1.9	120
20	F	44	7.7	1.9	125
21	M	74	4.9	0.99	150
22	F	73	7.3	1.9	200

TABLE 50b.

Serum Vitamin B_{12} Level (L.leichmannii assay) in Patients under Treatment for Pernicious Anaemia.

Case	Sex	Age	Hb.G/ 100 ml.	RBC Mill. /c.mm	Serum Vit. B_{12} level µµg/ml. ml.	Cyanocobalamin Last dose	Time since last dose	Total duration of Treatment	Usual dose given
23	F	64	12.0	4.6	170	100 µg.	5 mths	4 years	100 µg. /2 wks.
24	M	53	12.6	5.2	220	50 µg.	13 days	2 years	50 µg. /2 wks.
25	F	46	14.7	4.6	220	1 tab. Bifacton Orally	3 hrs.	6 mths.	2 tabs. daily
26	F	55	13.2	4.5	250	50 µg.	14 days	18 mths.	50 µg. /2 wks.
27	F	46	12.3	4.7	320	2 tab. Bifacton Orally	4 hrs.	11 mths.	4 tabs. daily
28	F	59	15.4	5.3	340	50 µg.	21 days	4 years	50 µg. /3 wks.
29	F	74	13.9	4.9	340	50 µg.	14 days	1 year	50 µg. /2 wks.
30	F	57	14.8	5.1	480	100 µg.	3 days	7 mths.	100 µg. /3 wks.
31	F	51	13.9	5.0	600	50 µg.	2 days	4 years	50 µg. /3 wks.
19	F	44	7.7	1.9	120	Untreated			
					1330	1 hour after 100 µg. IM			
					360	3 days after this injection			
					300	5 days after this injection			
			8.1	2.1	280	7 days after this injection			
20	F	43	7.7	1.9	125	Untreated			
					1520	1 hour after 100 µg. IM			
					290	2 days after this injection			
					205	4 days after this injection			
			8.0	2.2	160	6 days after this injection			

Note: Bifacton is a preparation of cyanocobalamin and intrinsic factor for oral administration. In all cases other than 25 and 27 the cyanocobalamin was given by injection.

TABLE 50c.

Serum Vitamin B_{12} Levels in Patients with Forms of Megaloblastic Anaemia other than Pernicious Anaemia.

Case	Sex	Age	Cause of megaloblastic anaemia	Hb.G./100 ml.	RBCs Mill. /c.mm.	Treatment before serum B_{12} test	Serum Vit. B_{12} level µµg./ml.
32	F	57	Partial Gastrectomy	9.5	4.3	PGA	110
33	M	52	Idiopathic * steatorrhoea	15.3	5.1	Nil	120
34	M	53	Idiopathic * steatorrhoea	11.8	3.8	PGA	140
35	F	33	Pregnancy	4.2	1.0	Nil	160
36	F	35	Pregnancy	4.9	2.0	Nil	160
37	M	28	Idiopathic steatorrhoea	7.0	3.1	Nil	160
38	M	42	Idiopathic steatorrhoea	13.3	5.3	PGA	160
39	M	21	Adult coeliac	12.5	3.7	Nil	160
40	F	33	Pregnancy	8.2	2.1	Nil	170
41	F	23	Pregnancy	10.2	3.1	Nil	180
42	F	47	Regional jejunitis	10.1	3.7	Nil	180
43	F	38	Pregnancy	8.9	3.5	Nil	190
44	M	45	? Abdominal tuberculosis	9.8	3.3	PGA	210
45	F	27	Pregnancy	4.3	1.5	Nil	240
46	F	35	Pregnancy	5.9	1.5	Nil	250
47	F	40	Pregnancy	12.3	4.9	PGA	250
48	F	30	Pregnancy	9.9	2.5	Nil	260
49	F	32	Pregnancy	7.3	2.7	Nil	260
50	M	53	Idiopathic* steatorrhoea	14.3	4.7	Nil	290
51	F	48	Idiopathic* steatorrhoea	13.5	4.7	PGA	310
52	F	43	Idiopathic steatorrhoea	13.0	5.1	PGA B_{12} 16 mth.prev.	370
53	M	26	Idiopathic steatorrhoea	14.2	5.3	PGA B_{12} till 3 weeks previously	390
54	F	37	Idiopathic steatorrhoea	13.6	4.3	B_{12} 10 days previously	430
55	F	41	Pregnancy	13.9	4.6	Anaemia was 7 months before; no PGA for 6 months	530

* In these 4 cases there was evidence of folic acid malabsorption but no megaloblastic change in the marrow. Cases 34 and 51 had had prolonged PGA therapy.

TABLE 50d.

Serum Vitamin B_{12} Levels (L.leichmannii Assay) in Various Conditions.

Case	Sex	Age	Diagnosis	Hb. G. /100 ml.	RBC Mills./ c.mm.	Serum Vit. B_{12} level µµg./ml.
56	F	57	? Subacute comb.degen.	10.2	2.9	< 50
57	F	73	Macrocytic anaemia	10.1	2.7	50
58	F	39	Iron defic. anaemia	8.3	4.4	140
59	F	67	Post gastrectomy	13.6	4.9	140
60	M	33	Lymphadenoma	6.7	3.3	160
61	F	39	Iron defic. anaemia	4.1	3.6	160
62	M	65	Partial gastrectomy	10.2	3.0	170
63	M	25	Diarrhoea	13.5	4.5	170
64	F	75	Iron defic. anaemia	4.4	2.8	190
65	F	45	Iron defic. anaemia	8.1	5.1	200
66	F	33	Iron defic. anaemia	9.2	4.1	200
67	F	25	Disseminated sclerosis	12.1	4.2	220
68	F	46	Iron defic. anaemia	11.7	4.5	230
69	M	29	Normal	15.4	5.3	230
70	F	30	Pregnant	6.5	4.3	230
71	F	45	Refract. iron defic.anaemia	14.1	4.9	230
72	M	34	Hepatic cirrhosis	10.4	4.1	230
73	F	42	Peripheral neuritis	13.9	4.7	230
74	F	47	Iron defic. anaemia	9.5	4.1	240
75	M	32	Lymphadenoma	7.5	3.9	240
76	M	47	Partial gastrectomy	15.1	5.2	250
77	F	61	Glossitis	11.8	3.8	250
78	F	51	Iron defic. anaemia	5.6	4.3	250
79	M	31	Haemolytic anaemia	9.5	2.6	250

Case	Sex	Age	Diagnosis	Hb. G. /100 ml.	RBC Mills. /c.mm.	Serum Vit. B_{12} level µµg./ml.
80	F	53	Partial gastrectomy	11.8	3.8	250
81	F	53	Rheumatoid arthritis	11.5	4.1	260
82	F	49	Haemolytic anaemia	10.8	3.6	270
83	F	35	Iron defic. anaemia	6.7	4.6	300
84	F	34	Iron defic. anaemia	10.5	4.3	320
85	M	44	Intestinal short circuit	13.3	4.8	320
86	F	49	Peripheral neuritis	6.7	4.0	330
87	F	36	Iron defic. anaemia	9.3	4.4	350
88	M	34	Normal	15.2	5.4	380
89	F	54	Thyrotoxicosis	12.3	4.9	380
90	M	45	Coronary thrombosis	15.5	5.2	400
91	M	52	Neurosis	11.5	4.2	410
92	F	61	Glossitis	12.9	4.9	420
93	M	48	Amoebiasis	11.2	4.0	440
94	F	75	Carcinomatosis	3.3	1.4	440
95	M	59	Iron defic. anaemia	10.1	4.1	480
96	F	44	Iron defic. anaemia	12.4	4.2	500
97	F	55	Myxoedema	13.9	4.9	500
98	F	57	Coronary thrombosis	14.6	5.1	500
99	F	42	Iron defic. anaemia	11.0	4.0	550
100	F	62	Idiopathic thrombocyth-aemia	5.8	2.4	550
101	M	59	Coronary thrombosis	15.1	5.2	550
102	F	37	Iron defic. anaemia	8.3	4.6	600
103	F	73	Coronary thrombosis	13.2	-	750

It will be seen from tables 50a, b, c, d, that the serum vitamin B_{12} level in pernicious anaemia in relapse is usually less than 130 μμg./ml. In Case 21, however, the level was higher than this, but there was nothing to suggest that the diagnosis was incorrect, and there was a response to injections of cyanocobalamin. By the Euglena assay (Dr. G.I.M.Ross) the level was 180 μμg./ml. In case 22 the level was even higher, but again there was nothing to suggest that the diagnosis was incorrect, and the patient responded to cyanocobalamin therapy in the usual way. The effect of alkaline hydrolysis was not tried, since although this can be carried out on pooled sera, it is not possible to manipulate very small quantities of serum satisfactorily in order to do this. Cases 32-55 were suffering from various forms of megaloblastic anaemia. Some were untreated, several were receiving pteroylglutamic acid, and one (case 53) was being treated with cyanocobalamin by injection and pteroylglutamic acid by mouth. The level was in the pernicious anaemia range in one case of untreated intestinal malabsorption (Case 33: also shown as Case 13, p.360) and one of megaloblastic anaemia after partial gastrectomy (Case 32: also shown as Case 201, p.533). In the other patients the level ranged from 140 to 750 μμg./ml., except that in two untreated patients who had clinical features suggesting pernicious anaemia, but a normoblastic marrow, the level was 50 μμg./ml. or less. In one of these cases the result was confirmed by Dr. Ross using the Euglena method. In the other there was insufficient pre-treatment serum for him to perform an assay. The details of these cases/

cases are given later on pp.424 and 433.

The results on the treated pernicious anaemia patients suggest that the maintenance therapy being given to them is adequate. It may well be that once the tissue stores are replenished by adequate treatment, the serum vitamin B_{12} level is more easily kept within normal limits by the usual maintenance dosage of cyanocobalamin than it is in the same patient receiving the same dosage in the early stages of treatment when the tissue stores are empty. This can no doubt be answered by giving a series of pernicious patients say 100 µg. of cyanocobalamin every three weeks by injection and estimating the serum vitamin B_{12} level on the day before each injection over a long period.

In conclusion it may be said that the difficulty about the Euglena assay is that the apparatus is complicated and the results of each individual assay are not available until more than a week has elapsed. On the other hand, the L.leichmannii assay is less sensitive and the measurement of the amount of vitamin B_{12} in the serum is just within its capacity and no more, particularly since the serum has to be diluted ten times in the course of its preparation for assay. Thus where the vitamin B_{12} reading is 80 µµg./ml., the amount actually added to a tube in the test, allowing for dilution of the serum, is 8 µµg./ml. or 0.000000000008 G./ml. The surprising feature is that any result is obtainable at all, and the findings given in Table 49 are felt to be particularly gratifying.

SUMMARY/

SUMMARY.

An account is given of a L.leichmannii method for assaying the serum level of vitamin B_{12}. In 19 out of 22 cases of untreated pernicious anaemia the level was less than 130 μμg./ml. The only other cases in which the level was not in the range 140 to 750 μμg./ml. were two patients with clinical features suggesting pernicious anaemia but with a normoblastic marrow, one case of intestinal malabsorption and one of megaloblastic anaemia after partial gastrectomy.